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(54) Title: METHODS FOR PREPARING LIPIDS/POLYNUCLEOTIDE TRANSFECTION COMPLEXES

(57) **Abstract**

Methods are provided for the preparation of transfection complexes of polynucleotides and cationic lipids suitable for delivering polynucleotides to cells. In particular, polynucleotide/cationic lipid transfection complexes are prepared in the absence of detergent by methods in which polynucleotides and cationic lipids are solubilized in an aqueous solution in the presence of co-solvent, and the removal of co-solvent under conditions which allow polynucleotide and cationic lipid to assemble into transfection complexes. The process produces a substantially homogenous population of tranfection complexes and is scaleable.

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# METHODS FOR PREPARING LIPID/POLYNUCLEOTIDE TRANSFECTION COMPLEXES

## Field of the Invention

This invention relates to preparation of polynucleotide transfection complexes and their use in delivering polynucleotides to cellshelper lipids, used in conjunction with cationic lipids, for the preparation of liposomes and other lipid-containing carriers of nucleic acids and other substances, for delivery to cells. In particular, the invention relates to methods for preparing complexes of polynucleotides and cationic lipids suitable for transfecting eukaryotic cells *in vivo* and *in vitro*.

## Background of the Invention

A number of methods exist for introducing exogenous genetic material to cells, which methods have been used for a wide variety of applications including, for example, research uses to study gene function, and ex vivo or *in vivo* genetic modification for therapeutic purposes. Ex vivo genetic modification involves the removal of specific cells from an animal, including humans, introduction of the exogenous genetic material, and then re-introduction of the genetically modified cells into the animal. By contrast, *in vivo* genetic modification involves the introduction of genetic material directly to the animal, including humans, using an appropriate delivery vehicle, where it is taken up directly by the target cells.

Generally, the various methods used to introduce nucleic acids into cells have as a goal the efficient uptake and expression of foreign genes. In particular, the delivery of exogenous nucleic acids in humans and/or various commercially important animals will ultimately permit the prevention, amelioration and cure of many important diseases and the development of animals with commercially important characteristics. The exogenous genetic material, either DNA or RNA, may provide a functional gene which, when expressed, produces a protein lacking in the cell or produced in insufficient amounts, or may provide an antisense DNA or RNA or ribozyme to interfere with a cellular function in, e.g., a virus-infected cell or a cancer cell, thereby providing an effective therapeutic for a disease state.

Cationic lipids have been developed that greatly facilitate nucleic acid delivery to cells, both *in vitro* and *in vivo*. See, for example, U.S. Patent No. 5,264,618, which describes techniques for using lipid carriers, including the preparation of liposomes and pharmaceutical compositions and the use of such compositions in clinical situations. A number of cationic lipids have been developed, which are generally amphipathic molecules comprising a positively charged headgroup, varying from single to multiple positive charges, linked to hydrophobic lipid tail groups or steroidal groups.

For cationic lipid-mediated delivery, the cationic lipids typically are mixed with a non-cationic lipid, usually a neutral lipid, and allowed to form stable liposomes, which liposomes are then mixed with the nucleic acid to be delivered. The liposomes may be large unilamellar vesicles (LUVs), multilamellar vesicles (MLVs) or small unilamellar vesicles (SUVs). The liposomes are mixed with nucleic acid in solution, at concentrations and ratios optimized for the target cells to be transfected, to form cationic lipid-nucleic acid transfection complexes. Alterations in the lipid formulation allow preferential delivery of nucleic acids to particular tissues *in vivo*. See PCT patent application numbers WO 96/40962 and WO 96/40963.

Nucleic acids are generally large polyanionic molecules which, therefore, bind cationic lipids and other positively-charged carriers through charge interactions. It is believed that the positively charged carriers such as cationic lipids form tight complexes with the nucleic acid, thereby condensing it and protecting it from nuclease degradation. In addition, cationic lipid carriers may act to mediate transfection by improving association with negatively-charged cellular membranes by giving the complexes a positive charge, and/or enhancing transport from the cytoplasm to the nucleus where DNA may be transcribed.

With respect to any of the cationic lipid carriers, transfection efficiency is highly dependent on the characteristics of the cationic lipid/nucleic acid complex. The nature of the complex that yields optimal transfection efficiency depends upon the mode of delivery, e.g. ex vivo or *in vivo*; for *in vivo* delivery, the route of administration, e.g., intravenous, intraperitoneal, inhalation, etc.; the target cell type, etc. Depending on the use, therefore, different carriers will be preferred. In addition to the choice of cationic lipid carrier, transfection efficiency will depend on certain physical characteristics of the complexes as well, such as charge and size. In addition,

the stability of the complexes during storage will be highly dependent on the physical nature of the complexes.

These characteristics depend largely on the method by which the complexes are prepared. Particularly for human therapeutic purposes, therefore, it is desirable to have a method of forming the nucleic acid/polycationic carrier complexes in a highly controllable manner. Further, it is desirable to have a process for preparing the complexes which is highly reproducible and scaleable.

The present invention provides these and related advantages as well.

Relevant Literature

Cationic lipid carriers have been shown to mediate intracellular delivery of plasmid DNA (Felgner et al., (1987) Proc. Natl. Acad. Sci. (USA), 84:7413-7416); mRNA (Malone et al., (1989) Proc. Natl. Acad. Sci. (USA) 86:6077-6081); and purified transcription factors (Debs et al., (1990) J. Biol. Chem. 265:10189-10192), in functional form. Literature describing the use of lipids as carriers for DNA include the following: Zhu et al., (1993) Science, 261:209-211; Vigneron et al., (1996) Proc. Natl. Acad. Sci. USA, 93:9682-9686; Hofland et al., (1996) Proc. Natl. Acad. Sci. USA, 93:7305-7309; Alton et al., (1993) Nat. Genet. 5:135-142; von der Leyen et al., (1995) Proc. Natl. Acad. Sci. (USA), 92:1137-1141; See also Stribling et al., (1992) Proc. Natl. Acad. Sci (USA) 89:11277-11281, which reports the use of lipids as carriers for aerosol gene delivery to the lungs of mice. For a review of liposomes in gene therapy, see Lasic and Templeton, (1996) Adv. Drug Deliv. Rev. 20:221-266.

The role of helper lipids in cationic lipid-mediated gene delivery is described in Felgner et al., (1994) J. Biol. Chem. 269(4): 2550-2561 (describing improved transfection using DOPE); and Hui et al., (1996) Biophys. J. 71: 590-599. The effect of cholesterol on liposomes *in vivo* is described in Semple et al., (1996) Biochem. 35(8): 2521-2525.

A method of preparing cationic lipid/nucleic acid transfection complexes by first forming lipid micelles in the presence of detergent is described in WO 96/37194. The effect of surfactants on DNA/lipid complexes and transfection activity are described in Liu et al., (1996) Pharm. Res. 13(11):1642-1646. The effect of liposome preparation and complex size on cationic lipid-mediated gene delivery is described in Templeton et al., (1997) Nat. Biotech. 15(7):647-652. The use of polyethylene glycol

in DNA/lipid complexes for aerosol delivery is described in Eastman et. al., (1997) Hum. Gene Ther. 8(6):765-773.

Summary of the Invention

The invention provides a method of preparing a cationic lipid/polynucleotide transfection complex, the method comprising co-solubilizing a cationic lipid carrier and a polynucleotide in an aqueous solution in the presence of a co-solvent, then removing the co-solvent such that the polynucleotide and cationic lipid carrier molecules "nucleate" in solution, forming an aqueous dispersion of cationic lipid/polynucleotide transfection complexes. The polynucleotide, cationic lipid and, optionally, non-cationic lipid, are co-solubilized in the ratios desired in the final transfection complex, which ratios will be dependent upon the lipids used, the target cell type and, if administered *in vivo*, the route of administration.

The polynucleotide is typically plasmid DNA, generally including a recombinant expression construct, the DNA encoding a transcription product and operatively linked regulatory elements, whereby the DNA is capable of transcription in the target cells. As used herein, the term "transcription product" is intended to encompass an RNA product resulting from transcription of a nucleic acid sequence, and includes RNA sequences that are not translated into protein (such as antisense RNA or ribozymes) as well as RNAs that are subsequently translated into polypeptides or proteins. Also included is the preparation of complexes including polycationic carriers and oligonucleotides.

In preferred embodiments, the cationic lipid is DOTIM, the neutral lipid is cholesterol, and the lipid and DNA are co-solubilized in a solution of about 70% ethanol and 10% ethyl acetate. Preferably, the co-solvents are removed by dialysis, and the resulting cationic lipid/polynucleotide transfection complexes have an average size of less than about 500 nm.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

Brief Description of the Drawings

Figure 1 shows the density gradient profiles of DNA:cationic lipid complexes. Profiles are measured by flow-cell UV spectrophotometer at 237nm, which is the absorbance of DOTIM. The contents of the centrifuged samples (approx. 13ml) are pumped through the flow-cell at a rate of 1 ml/min. The ordinate represents the approximate location within the centrifuge tube. From top to bottom Figure 1 shows the density gradient profiles of complex preparations: a) prepared by the standard mixing method in a 1:5 DNA/cationic lipid ratio (μg DNA:nmoles cationic lipid); b) prepared by the solution nucleation method in a 1:1 DNA/cationic lipid ratio; and c) prepared by the standard mixing method in a 1:1 DNA/cationic lipid ratio.

Description of Specific Embodiments

The physical nature of polynucleotide/cationic lipid transfection complexes is highly dependent on the method in which they are prepared. The physical nature of the transfection complexes, in turn, is important for the ability of the complexes to transfect target cells. In addition, the stability of the complexes during storage is highly dependent upon their physical nature. Typically, more homogenous compositions are more stable during storage than heterogeneous compositions. Also, compositions containing a higher proportion of active particles utilize starting materials more efficiently, and, in many cases less material will be necessary to achieve desired transfection rates, thereby decreasing any undesirable toxicities associated with excess material.

Typically, transfection complexes are prepared by adding one solution to the other, i.e. nucleic acid to pre-formed liposomes or liposomes to nucleic acid, with constant stirring. Transfection complexes prepared by prior art methods of adding one solution to the other results in a heterogeneous mix of complexes because the environment under which the complexes are formed is constantly changing. In addition, most prior art methods of complex preparation involve first preparing the lipids in a dispersed liposomal form. Thus, most cationic lipid carriers are formulated with a neutral lipid to aid in forming stable liposomal intermediates. The lipids are usually dried as a film in an organic solution such as chloroform, then dispersed in an aqueous solution. In an aqueous environment, the lipids spontaneously form liposomes. The liposomes are formed as a heterogeneous mix unilamellar and multilamellar vesicles, in a range of sizes. They are then usually sonicated or

extruded through membranes with specified pore sizes, usually to form small unilamellar vesicles (SUVs). See, e.g., *Liposome Technology* (CFC Press, NY 1984); *Liposomes* by Ortro (Marcel Schher, 1987); *Methods Biochem Anal.* 33:337-462 (1988). By contrast, the method of the present invention avoids the preparation and use of liposomal intermediates.

The method of the present invention allows the formation of nucleic acid/cationic lipid transfection complexes by a process termed "solution nucleation." In short, the process involves the co-solubilization of the desired components in the ratios desired in the final transfection complex. The solution will contain an aqueous component and one or more co-solvents. Upon co-solubilization, the lipid and polynucleotide components are substantially miscible within the solution. The co-solvent(s) may then be removed, resulting in a substantially aqueous environment. The loss of hydration within the co-solvent(s) may cause the polynucleotides to compact or condense more readily than within a more aqueous environment.

Upon co-solvent removal, individual components will seek a more favorable physical state by assembling into lipid/polynucleotide complexes. They will form complexes by physical interactions such as electrostatic interactions between the cationic lipid and the polynucleotide, hydrophobic interactions between the lipid components, van der Waals forces, etc. Each point where the components assemble into complexes is essentially a "nucleation" event. The method results in all complexes being formed in essentially identical environments, where each component "sees" the other components in the same ratios under the same conditions.

"Transfection" as used herein means the delivery of exogenous nucleic acid molecules to a cell, either *in vivo* or *in vitro*, whereby the nucleic acid is taken up by the cell and is functional within the cell. A cell that has taken up the exogenous nucleic acid is referred to as a "host cell", "target cell" or "transfected cell." A nucleic acid is functional within a host cell when it is capable of functioning as intended. Usually, the exogenous nucleic acid will comprise an expression cassette which includes DNA coding for a gene of interest, with appropriate regulatory elements, which will have the intended function if the DNA is transcribed and translated, thereby causing the host cell to produce the peptide or protein encoded therein. DNA may encode a protein lacking in the transfected cell, or produced in insufficient quantity or less active form, or secreted, where it may have an effect on cells other

than the transfected cell. Other examples of exogenous nucleic acid to be delivered include, e.g., antisense oligonucleotides, mRNA ribozymes, or DNA encoding antisense RNA or ribozymes. Nucleic acids of interest also include DNA coding for a cellular factor which, when expressed, activates the expression of an endogenous gene.

"Transfection efficiency" refers to the relative number of cells of the total within a cell population that are transfected and/or to the level of expression obtained in the transfected cells. It will be understood by those of skill in the art that, by use of appropriate regulatory control elements such as promoters, enhancers and the like, the level of gene expression in a host cell can be modulated. The transfection efficiency necessary or desirable for a given purpose will depend on the purpose, for example the disease indication for which treatment is intended, and on the level of gene expression obtained in the transfected cells.

"Polycation" refers to any molecular entity having multiple positive charges. Because of their positive charges, polycations interact electrostatically with negatively charged polynucleotides, usually condensing the polynucleotide molecules. "Polycationic carrier" refers to a polycation which, when combined with a polynucleotide, forms a complex suitable for transfecting eukaryotic cells. Cationic lipids have been shown to be efficient polycationic carriers for nucleic acid delivery to cells. Typically, cationic lipid carriers are formulated with both cationic and non-cationic lipid (usually neutral lipid) components. Thus, a "lipid carrier" or "cationic lipid carrier" refers to a lipid composition of one or more cationic lipids and, optionally, one or more non-cationic lipids for delivering agents to cells. A lipid carrier may be complexed with other polycations or additional transfection facilitating agents.

"Transfection complex" or "polynucleotide transfection complex" refers to a combination of a polycationic carrier and a nucleic acid, in any physical form, for use in transfecting eukaryotic cells. A transfection complex may include additional moieties, e.g., targeting molecules such as receptor ligands or antibody fragments, or other accessory molecules. For example, nuclear localizing peptides may be included for facilitating transport of the polynucleotide to the cell nucleus. Kalderon et al., (1984) Cell 39:499-509; Chelsky et al., (1989) Mol. Cell Biol. 9:2487-2492; Dingwall & Laskey (1991) Trends Biochem. Sci. 16:478-481. Proteins or peptides may be

included in the transfection complex to facilitate release of the transfection complex from the endosome after internalization. Raja-Walia et al., (1995) Hum. Gene Therap. 2:521-530; Bai et al., (1993) J. Virol. 67:5198-5205. In addition, enzymes involved in transcription and/or translation may be included to facilitate gene expression in the cell cytoplasm without transport to the cell nucleus. Gao & Huang (1993) Nucl Acids Res. 21:2867-2872.

The transfection complexes may also be prepared to include a targeting moiety, to specifically deliver complexes to the desired target cell *in vivo*. Thus, strategies are known in the art for including receptor ligands for delivery to cells expressing the appropriate receptor, or using antibodies or antibody fragments to target transfection complexes to cells expressing a specific cell surface molecule. See WO 96/37194; Ferkol et al., (1993) J. Clin. Invest. 92:2394-2400.

The term "cationic lipid" is intended to encompass lipids that are positively charged at physiological pH, and more particularly, constituitively positively charged lipids comprising, for example, a quarternary ammonium salt moiety. Cationic lipids used for gene delivery typically consist of a hydrophilic polar head group and lipophilic aliphatic chains. Alternatively, cholesterol derivatives having a cationic polar head group are used in a similar manner. Farhood et al., (1992) Biochim. Biophys. Acta 1111:239-246; Vigneron et al., (1996) Proc. Natl. Acad. Sci. (USA) 93:9682-9686.

Cationic lipids of interest include, for example, imidazolinium derivatives (WO 95/14380), guanidine derivatives (WO 95/14381), phosphatidyl choline derivatives (WO 95/35301), and piperazine derivatives (WO 95/14651). Examples of cationic lipids that may be used in the present invention include DOTIM (also called BODAI) (Solodin et al., (1995) Biochem. 34: 13537-13544), DDAB (Rose et al., (1991) BioTechniques 10(4):520-525), DOTMA (U.S. Patent No. 5,550,289), DOTAP (Eibl and Wooley (1979) Biophys. Chem. 10:261-271), DMRIE (Felgner et al., (1994) J. Biol. Chem. 269(4): 2550-2561), EDMPC (commercially available from Avanti Polar Lipids, Alabaster, Alabama), DC-Chol (Gau and Huang (1991) Biochem. Biophys. Res. Comm. 179:280-285), (Behr et al., (1989) Proc. Natl. Acad. Sci. USA, 86:6982-6986), MBOP (also called MeBOP) (WO 95/14651), and those described in WO 97/00241.

Particularly preferred are EDMPC for aerosolized delivery to airway epithelial cells, and DOTIM, DOTAP or MBOP for intravenous delivery to vascular endothelial cells of various organs, particularly the lung. In addition, cationic lipid carriers having more than one cationic lipid species may be used to produce complexes according to the method of the present invention.

Non-cationic lipids of use in transfection complexes are known, and include, for example, dioleoyl phosphatidylethanolamine (DOPE), Hui et al., (1996) Biophys. J. (71):590-599; cholesterol, Liu et al., (1997) Nat. Biotech. (15):167-173; and dilauroyl phosphatidylethanolamine (DLPE) (co-pending patent application serial no. 08/832,749). Normally, cationic lipid and non-cationic lipids are used in approximately equimolar amounts, but need not be.

Additional polycationic carriers include positively charged peptides and proteins, both naturally occurring and synthetic, as well as polyamines, carbohydrates or synthetic polycationic polymers. Examples include polylysine, polyarginine, protamine, polybrene, histone, cationic dendrimer, and synthetic polypeptides based on viral peptides, e.g., having cell binding, endosomal release or nuclear localizing functions, etc. For certain applications, polycationic carriers may include cationic lipid as well as peptide moieties. See, e.g., WO 96/22765.

The nucleic acid may be in any physical form, e.g., linear, circular or supercoiled; single-stranded, double-, triple-, or quadruple-stranded; and further including those having naturally occurring nitrogenous bases and phosphodiester linkages as well as non-naturally occurring bases and linkages, e.g. for stabilization purposes. Preferably it is in the form of supercoiled plasmid DNA. Plasmid DNA is conveniently used for DNA transfections since there are no size constraints on the DNA sequences that may be included, and it can be produced in large quantity by growing and purifying it from bacterial cells.

Polynucleotide transfection complexes are formed by the electrostatic binding between the polynucleotide and the polycationic carrier. In addition to the mixing conditions, the physical structure of such complexes depends on the polycationic carrier and nucleic acid components, the ratios between them, concentrations of each, buffer ionic strength, and the like. Smith et al., (1997) Adv. Drug Deliv. Rev. 26:135-150.

Initially, the components of the transfection complex must be co-solubilized in a solution in the ratios desired in the final complex. The solution will contain water and one or more co-solvents. As used herein, a co-solvent is any solvent other than water. Typically, co-solvents will include polar organic solvents. The solution should be prepared in the absence of salts, which would decrease the solubility of the lipid and polynucleotide components and interfere with the complexation process.

The co-solvent is usually one that is miscible with water and, when combined with the aqueous component, the polynucleotide and lipids are both soluble. The selection of co-solvent will depend on the lipid(s) used. Solubility of many lipids are known or can be determined from, for example, CRC Handbook of Chemistry and Physics, (76th edition, CRC Press, New York, David R. Lide editor), Merck Index (Merck & Co., Inc., Whitehouse Station, New Jersey, 1996) or from the catalogue of lipid vendors such as Avanti Polar Lipids (Birmingham, Alabama). If more than one lipid component is used, for example a cationic lipid and a neutral lipid, the least soluble lipid is the preferred starting point for selecting a co-solvent or combination of co-solvents. Once the solubility of the individual components is determined, the choice of co-solvents and relative amounts may be determined empirically.

Where the resulting transfection complexes are to be administered *in vivo* to humans, the solvents should be selected to minimize any safety or regulatory issues. Short chain alcohols are useful co-solvents because nucleic acids remain soluble up to high levels. A preferred alcohol is ethanol. Other useful co-solvents include chloroform, methanol, propanol, butanol (e.g. *t*-butanol), pentanol, ethyl acetate, ether, pyridine, benzene, polyethylene glycol (MW 1000 to 20,000), dimethyl sulfoxide (DMSO), carbon tetrachloride, phenol, benzene, methylene chloride, acetonitrile, and miscible polymers such as polyvinylalcohol and polyvinylpyrrolidone Preferably, the components are co-solubilized in a solution containing at least about 50% ethanol and less than about 20% ethyl acetate, most preferably, the solution contains about 70% ethanol and about 10% ethyl acetate.

The components may be mixed in any order that avoids precipitation, although the mixing process described in the examples that follow is preferred. The solution will be prepared in the absence of detergent. In a detergent solution, the lipid component will tend to form micelles. It is desirable to simplify the process to avoid issues relating to detergent removal.

Once the transfection components are co-solubilized into a single solution, the co-solvent is then carefully removed. During removal of the co-solvent, the least soluble component of the mixture will begin to come out of solution, causing a "nucleation" event. Upon continued removal of the co-solvent, lipid/polynucleotide complexes will form around such "nucleation" sites, resulting in an aqueous solution comprising substantially homogeneous lipid/polynucleotide transfection complexes.

The co-solvent is preferably removed by dialysis. Other means of co-solvent removal include, for example, diafiltration, tangential flow filtration, dilution, heating, freezing, etc. When removed by dialysis, any dialysis membrane may be used that is compatible with the solvent system used. A wide variety of dialysis membranes are available commercially, and solvent compatibility is generally available from the manufacturers' specifications. Preferably the dialysis membrane has the smallest molecular weight cutoff available. As an example, with an ethanol, ethyl acetate co-solvent system, a regenerated cellulose membrane with a molecular weight cutoff of about 12,000 to 14,000 daltons may be used, such as the Spectra/Por #2 dialysis membrane (Spectrum, Houston, Texas).

Dialysis will be performed against an aqueous solution. The dialysis buffer may be any buffer suitable for the subsequent uses of the complexes, and may include any physiologically acceptable buffer or no buffer. If the complexes will not be used immediately, but will be stored before use, the dialysis buffer selected will depend primarily on the lipid components of the complexes and will be of a composition and pH designed to preserve the stability of the complexes. Preferably, the dialysis buffer is a low ionic strength buffer to minimize interference by any additional ions in the complexation process. A low-ionic strength solution means a solution having a conductivity less than about 35 mS, preferably less than about 10 mS, and most preferably less than about 1 mS. Desirably, the dialysis solution will contain no salts. Where the resulting complexes will be used directly *in vivo*, a preferred dialysis buffer is 5% dextrose in 5 mM Tris-HCl (pH 8.0). Dialysis should be performed against a large excess of dialysis buffer, e.g., at least about 500-fold, and may be 1000-fold or greater.

If desired, the complexes may be concentrated after dialysis. The degree of concentration will depend on the desired use of the complexes, for example, any limitations in volume due to the intended route of administration. Methods for

concentration include, for example, vacuum dialysis, centrifugation, lyophilization, evaporation, and tangential flow filtration.

A number of analytical methods are known for characterizing the complexes prepared according to the method of the invention. Visual inspection may provide initial information as to aggregation of the complexes. Spectrophotometric analysis may be used to measure the optical density, giving information as to the aggregated state of the complexes; surface charge may be determined by measuring zeta potential; agarose gel electrophoresis may be utilized to examine the amounts and physical condition of the polynucleotide molecules in the complexes; particle sizing may be performed using commercially available instruments; HPLC analysis will give additional information as to resulting component ratios and any component degradation; and dextrose or sucrose gradients may be used to analyze the composition and heterogeneity of complexes formed.

The preferred size of the resulting complexes will depend on the desire use. For intravenous administration, the size is preferably less than about five microns, more preferably less than about 500 nm. For aerosol administration, the size should be less than about 500 nm, preferably around 100 nm or less. The size of the resulting complexes may be altered by adjusting the pH, the lipid:polynucleotide concentrations or ratios, the ratios of the lipid components, or by adjusting physical parameters such as temperature, viscosity or agitation as known for other nucleation processes. See, e.g, Mullin, J.W., Crystallization, 3rd Ed. (Butterworth-Heinemann, Oxford, 1993)

It will be appreciated that using the method of complex preparation described herein, polynucleotide transfection complexes may be prepared in a variety of formulations depending of the desired use. Uses contemplated for the complexes of the invention include both *in vivo* and *in vitro* transfection procedures corresponding to those presently known that use cationic lipid carriers, including those using commercial cationic lipid preparations, such as Lipofectin(, and various other published techniques using conventional cationic lipid technology and methods. See, generally, Lasic and Templeton (1996) Adv. Drug Deliv. Rev. 20: 221-266 and references cited therein. Thus, the ratios of each component in the complexes, final concentrations, buffer solutions, and the like are easily adjusted by adjusting the starting components. The method allows the resulting transfection complexes to the

prepared in a highly controlled fashion, efficiently using starting materials and yielding active transfection complexes.

Cationic lipid-nucleic acid transfection complexes can be prepared in various formulations depending on the target cells to be transfected. See, e.g., WO 96/40962 and WO 96/40963. While a range of lipid/polynucleotide complex formulations will be effective in cell transfection Since the activity of a given cationic lipid-nucleic acid transfection complex in transfecting cells *in vitro* does not correlate with *in vivo* activity, optimum conditions are determined empirically in the desired experimental system. Lipid carrier compositions may be evaluated by their ability to deliver a reporter gene (e.g. CAT which encodes chloramphenicol acetyltransferase, luciferase, or (-galactosidase) *in vitro*, or *in vivo* to a given tissue in an animal, such as a mouse.

For *in vitro* transfections, the various combinations are tested for their ability to transfect target cells using standard molecular biology techniques to determine DNA uptake, RNA and/or protein production. Typically, *in vitro* cell transfection involves mixing nucleic acid and lipid, in cell culture media, and allowing the lipid-nucleic acid transfection complexes to form for about 10 to 15 minutes at room temperature. The transfection complexes are added to the cells and incubated at 37°C for about four hours. The complex-containing media is removed and replaced with fresh media, and the cells incubated for an additional 24 to 48 hours.

*In vivo*, particular cells can be preferentially transfected by the use of particular cationic lipids for preparation of the lipid carriers, for example, by the use of EDMPC to transfect airway epithelial cells (WO 96/40963) or by altering the cationic lipid-nucleic acid formulation to preferentially transfect the desired cell types (WO 96/40962). Thus, for example, in circumstances where a negatively charged complex is desired, relatively less cationic lipid will be complexed to the nucleic acid resulting in a higher nucleic acid: cationic lipid ratio. Conversely, in circumstances where a positively charged complex is desired, relatively more cationic lipid will be complexed with the nucleic acid, resulting in a lower nucleic acid: cationic lipid ratio.

The lipid mixtures are complexed with DNA in different ratios depending on the target cell type, generally ranging from about 6:1 to 1:20 μg DNA:nmole cationic lipid. For transfection of airway epithelial cells, e.g., via aerosol, intratracheal or intranasal administration, net negatively charged complexes are preferred. Thus, preferred DNA:cationic lipid: DNA ratios are from about 10:1 to about 1:20,

preferably about 3:1. For intravenous administration, preferred DNA:cationic lipid: DNA ratios range from about 1:3 to about 1:20 μg DNA: nmole cationic lipid, most preferably, about 1:6 to about 1:15 μg DNA: nmole cationic lipid. Additional parameters such as nucleic acid concentration, buffer type and concentration, etc., will have an effect on transfection efficiency, and can be optimized by routine experimentation by a person of ordinary skill in the art.

Delivery can be by any means known to persons of skill in the art, e.g., intravenous, intraperitoneal, intratracheal, intranasal, intramuscular, intradermal, etc. PCT patent application WO 96/40962 describes the preparation and use of cationic lipid carriers for *in vivo* DNA delivery. For aerosol administration, via intranasal or intraoral delivery, the cationic lipid-nucleic acid transfection complex will withstand both the forces of nebulization and the environment within the lung airways and be capable of transfecting lung cells. Techniques for delivering genes via aerosol administration of cationic lipid-DNA transfection complexes is described in U.S. Patent No. 5,641,662.

The following examples are offered by way of illustration and not by way of limitation.

## EXAMPLES

### Example 1: Complex Preparation by Solution Nucleation

Plasmid p4119 containing the CAT reporter gene under the control of the HCMV promoter was prepared at a concentration of 8.2 mg/ml in 10 mM Tris, pH 8.0. 100 mg cholesterol (Sigma, St. Louis, MO) and 100 mg DOTIM (Sigma) were each dissolved into 5 ml ethyl acetate solutions, making 20 mg/ml solutions (final concentrations of 28.75 mM DOTIM and 51.7 mM cholesterol).

The DNA/lipid solution was prepared in a 4 ml glass vial by mixing 1400 μl EtOH, 321 μl water, and 76.2 μl DNA. Then 165.16 μl ethyl acetate was added, followed by addition of 12.1 μl 51.7 mM cholesterol and 21.74 μl 28.75 mM DOTIM. The final solution contained 70% EtOH and 10% ethyl acetate; 3.125 mM DOTIM and 3.125 mM cholesterol for a 1:1 molar ratio; and DNA in a final concentration of 0.3125 mg/ml for a 1:1 DNA:cationic lipid ratio The co-solubilized DNA/lipid solution was dialyzed against 1 liter of 5% dextrose, 10 mM Tris, pH 8.0 for four

hours usinga Spectra/Por#2 dialysis membrane (regenerated cellulose: MW cuoff 12Kd-14 Kd)

DNA/lipid complexes were also prepared by standard mixing methods as follows. Liposomes were prepared by first dissolving the lipids (DOTIM and cholesterol) in a mixture of chloroform and methanol (1:1 molar ratio) and lipid films were formed with a rotary evaporator. The films were hydrated with 5% dextrose in water (D5W) at room temperature and the resulting liposomes extruded through a series of membranes having pore sizes of 400nm, 200nm, and 50nm.

DNA-liposome complexes were prepared at a 1:5 DNA:cationic lipid ratio (mg DNA:umole cationic lipid) by adding the DNA, in a solution at 0.625 mg/ml concentration in D5W to the solution containing liposomes, in an equal volume, with constant stirring, using a Hamilton Dilutor 540B (Hamilton, Reno, Nevada). DNA-liposome complexes were also prepared at a 1:1 DNA:cationic lipid ratio (mg DNA:umole cationic lipid) in a similar manner except that the solution containing liposomes was added to the solution containing the DNA.

Example 2: Characterization of DNA/lipid complexes

DNA/lipid complexes were sized using a NiComp 370 particle sizer and found to be 135 nm ( 78 in diameter. HPLC analysis was performed using a 10 µl sample, analyzed on a Shimadzu LC-10AD HPLC equipped with an Altima C8 column, 250 cm x 4.6 mm, ID 5 (m, Model #88075. The column was previously equilibrated with a mobile phase consisting of 65% acetonitrile, 25% v/v methanol, 9.9% v/v water, and 0.1% v/v trifluoroacetic acid. Following injection, mobile phase is run at a rate of 1 ml/min, 37(C for 20 minutes. Elution peaks are detected by UV absorbance at 215 nm.

The results showed the final complexes contained 42.3% DOTIM and 42.2% cholesterol, maintaining the original 1:1 molar ratio. By agarose gel electrophoresis, plasmid DNA was visible in its native forms.

Dextrose gradients (5% w/v to 20% w/v) were prepared using the BioComp Gradient Master (BioComp Instruments, Inc., New Brunswick, Canada). At room temperature, centrifuge tubes (14 x 89 mm) were half-filled with 5% dextrose followed by careful addition of 6 ml of 20% dextrose to the bottom of the tube with a syringe and canula. The tubes were placed in the Gradient Master and programmed to produce the linear gradients (time = 2 min 25 sec., angle = 81.5°, speed setting = 15).

The gradients were allowed to equilibrate to 4(C for about 4 hrs. Approximately 300 µl sample was loaded to the top of the gradient, and spun for 2 hrs at 40,000 rpm and 4°C using a Beckman XL-70 ultracentrifuge with a SW-41 rotor. The centrifuged gradients were loaded into a tube piercing apparatus (Brandell) and 50% w/v dextrose was pumped at 30 ml/min into the bottom of the tube. The contents of the tube were forced through an on-line UV/VIS spectrophotometer (Rainin) and absorbance was measured at 237nm (DOTIM absorbance). The data was analyzed using Rainin HPLC Dynamax software run on an Apple Macintosh Quadra 610.

Figure 1 shows the density gradient profile of DNA/lipid complexes prepared according to the methods described above. From top to bottom, Figure 1 shows the density gradient profiles of complex preparations: a) prepared by the standard mixing method in a 1:5 DNA/cationic lipid ratio (µg DNA:nmoles cationic lipid); b) prepared by the solution nucleation method in a 1:1 DNA/cationic lipid ratio; and c) prepared by the standard mixing method in a 1:1 DNA/cationic lipid ratio.. The density gradient profile shows that the solution nucleation method produces a less heterogeneous mixture of DNA-lipid complexes than that prepared by standard mixing methods.

All publications and patent applications cited herein are hereby incorporated by reference to the same extent as if fully set forth herein.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

We claim:

1. A method of preparing a polynucleotide/cationic lipid transfection complex, said method comprising:

a) providing an aqueous solution comprising a cationic lipid, a polynucleotide, and a co-solvent, in the absence of a detergent, and

b) removing the co-solvent to form a polynucleotide/cationic lipid transfection complex.

2. The method according to claim 1 wherein the solution further comprises a non-cationic lipid.

3. The method according to claim 1 wherein the co-solvent is a polar organic solvent.

4. The method according to claim 1 wherein at least one co-colvent is selected from the group consisting of ethanol, chloroform, methanol, propanol, butanol, pentanol, ethyl acetate, ether, pyridine, benzene, polyethylene glycol, dimethyl sulfoxide, carbon tetrachloride, phenol, benzene, methylene chloride, acetonitrile, polyvinylalcohol and polyvinylpyrrolidone.

5. The method according to claim 3 wherein the solution comprises at least about 50% short-chain alcohol.

6. The method according to claim 5 wherein the short-chain alcohol is ethanol.

7. The method according to claim 1 wherein the polynucleotide is DNA.

8. The method according to claim 1 wherein the polynucleotide is RNA.

9 The method according to claim 7 wherein the DNA is plasmid DNA.

10. The method according to claim 1 wherein the solution further comprises a synthetic cationic polymer.

11. The method according to claim 1 wherein the solution further comprises a polypeptide.

12. The method according to claim 1 wherein the cationic lipid:polynucleotide ratio in the polynucleotide transfection complex is in the range of from about 10:1 to 1:20 μgram polynucleotide:nmole cationic lipid.

13. The method according to claim 1 wherein the cationic lipid is selected from the group consisting of DOTIM, DDAB, DOTMA, DOTAP, DMRIE, EDMPC, DC-Chol, and MBOP.

14. The method according to claim 2 wherein the non-cationic lipid is selected from the group consisting of DOPE, cholesterol and DLPE.

15. The method according to claim 1 wherein the polynucleotide transfection complex has an average size of less than about five microns.

16. The method according to claim 15 wherein the polynucleotide transfection complex has an average size of less than about 500 nanometers.

17. A polynucleotide transfection complex prepared according to the method of claim 1.

18. A method of delivering exogenous genetic capability to a eukaryotic cell comprising administering an effective amount of the polynucleotide transfection complex of claim 17.

19. The method according to claim 18 wherein said administering is *in vitro*.

20. The method according to claim 18 wherein said administering is *in vivo*.

21. The method according to claim 20 wherein said administering is by a method selected from the group consisting of intravenous, intraperitoneal, intradermal, subcutaneous, intramuscular, intratracheal, inhalation, topical, direct intra-organ injection and direct intratumoral injection.

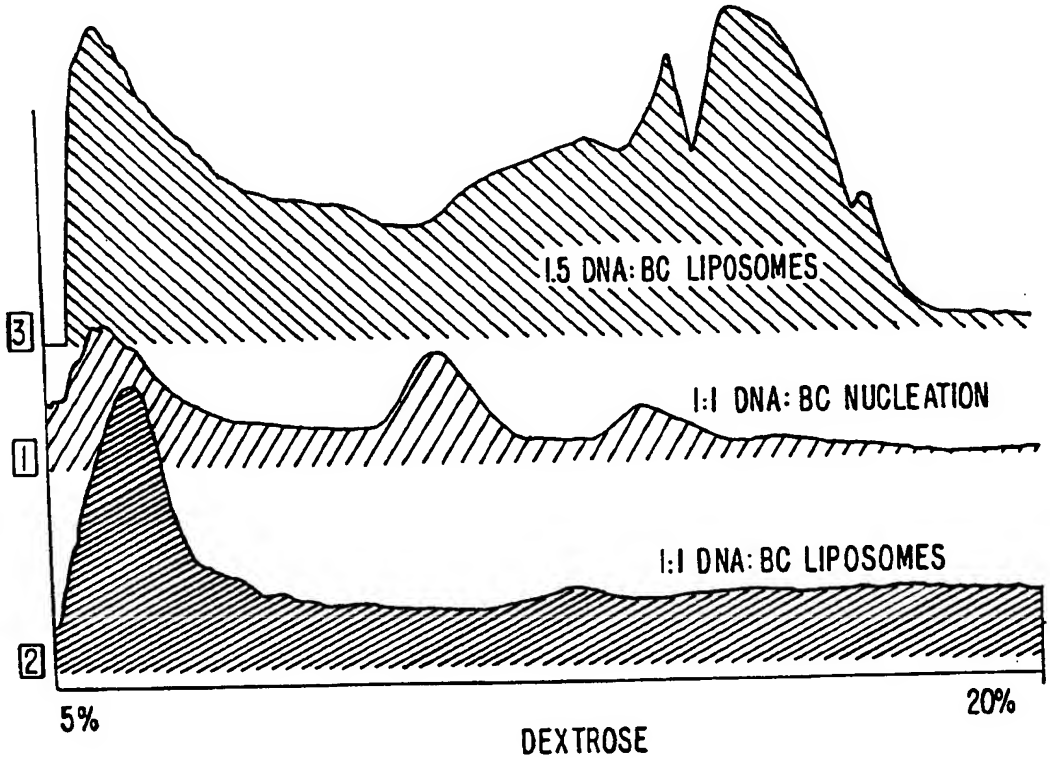
1.5 DNA: BC LIPOSOMES
1:1 DNA: BC NUCLEATION
1:1 DNA: BC LIPOSOMES
3
1
2
5%
20%
DEXTROSE

FIG. 1.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19936

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :Please See Extra Sheet.
US CL :264/4.1, 424/1.21, 417, 450; 514/812; 435/172.3; 514/44,12,2,3
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 264/4.1, 424/1.21, 417, 450; 514/812; 435/172.3; 514/44,12,2,3; 935/54

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, cation?, lipid#, liposome#, aqueous, cosolvent#, co(w)solvent#, ehthanol, etoh, methanol, meoh, chloroform, propanol

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 4,897,355 A (**EPPSTEIN et al**) 30 January 1990, see entire document. | 1-21 |
| Y | US 5,334,761 A (**GEBEYEHU et al**) 02 August 1994, see entire document. | 1-21 |
| Y | US 5,451,661 A (**WAN**) 19 September 1995, see entire document. | 1-21 |
| Y | **REIMER et al**, Formation of novel hydrophobic complexes between cationic lipids and plasmid DNA, Biochemistry, October 1995, Volume 34, pages 12877-12883. | 1-21 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | CAMPBELL et al, Lipofection reagents prepared by a simple ethanol injection technique, Chemical Abstracts, July 1995, Volume 123, No. 1, page 524, abstract no. 5027g, see entire document. | 1-21 |
| Y | KOIV et al, Influence of calcium and ethanol on the aggregation and thermal phase behavior of L-dihexadecylphosphatidylcholine liposomes, Chemical Abstracts, February 1993, Volume 118, No. 7, pages 327-328, abstract no. 54658u, see entire document. | 1-21 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19936

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 51/00, 38/00, 9/127, 31/70; A01N 25/26; B01J 13/02; C12N 15/00

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(54) Title: METHODS FOR THE MODULATION OF NEOVASCULARIZATION AND/OR THE GROWTH OF COLLATERAL ARTERIES AND/OR OTHER ARTERIES FROM PREEXISTING ARTERIOLAR CONNECTIONS

(57) **Abstract**

Described is the modulation of the neovascularization and/or growth of collateral arteries and/or other arteries from preexisting arteriolar connections. Methods are provided for enhancing neovascularization and/or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections comprising contacting organs, tissue or cells with a colony stimulating factor (CSF) or a nucleic acid molecule encoding said CSF. Furthermore, the use of a CSF or a nucleic acid molecule encoding said CSF for the preparation of pharmaceutical compositions for enhancing neovascularization and/or collateral growth of collateral arteries and/or other arteries from preexisting arteriolar connections is described. Also provided are methods for the treatment of tumors comprising contacting an organ, tissue or cells with an agent which suppresses neovascularization and/or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections through the inhibition of the biological activity of CSFs. Further described is the use of an agent which suppresses neovascularization and /or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections through inhibition of the biological activity of CFSs for the preparation of pharmaceutical compositions for the treatment of tumors.

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# Methods for the modulation of neovascularization and/or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections

The present invention relates generally to the modulation of neovascularization and/or the growth of collateral arteries or other arteries from preexisting arteriolar connections. In particular, the present invention provides a method for enhancing neovascularization and/or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections comprising contacting an organ, tissue or cells with a colony stimulating factor (CSF) or a nucleic acid molecule encoding said CSF. The present invention also relates to the use of a CSF or a nucleic acid molecule encoding said CSF for the preparation of pharmaceutical compositions for enhancing neovascularization and/or collateral growth of collateral arteries and/or other arteries from preexisting arteriolar connections. Furthermore, the present invention relates to a method for the treatment of tumors comprising contacting an organ, tissue or cells with an agent which suppresses neovascularization and/or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections through the inhibition of the biological activity of a CSF. The present invention further involves the use of an agent which suppresses neovascularization and/or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections through the inhibition of the biological activity of a CSF for the preparation of pharmaceutical compositions for the treatment of tumors.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including any manufacturer's specifications, instructions,

etc.) are hereby incorporated herein by reference; however, there is no admission that any document cited is indeed prior art as to the present invention.

In the treatment of subjects with arterial occlusive diseases most of the current treatment strategies aim at ameliorating their effects. The only curative approaches involve angioplasty (balloon dilatation) or bypassing surgery. The former carries a high risk of restenosis and can only be performed in certain arterial occlusive diseases, like ischemic heart disease. The latter is invasive and also restricted to certain kinds of arterial occlusive diseases. There is no established treatment for the enhancement of neovascularization and/or collateral growth.

Vascular growth in adult organisms proceeds via two distinct mechanisms, sprouting of capillaries (angiogenesis) and in situ enlargement of preexisting arteriolar connections into true collateral arteries (Schaper, J. Collateral Circulation - Heart, Brain, Kidney, Limbs. Boston, Dordrecht, London: Kluwer Academic Publishers; 1993). Recent studies have disclosed mechanisms leading to angiogenesis with vascular endothelial growth factor (VEGF) as a major component (Tuder, J. Clin. Invest. 95 (1995), 1798-1807; Plate, Nature 359 (1992), 845-848; Ferrara, Endocrine Reviews 13 (1992), 18-42; Klagsbrun, Annu. Rev. Physiol. 53 (1991), 217-239; Leung, Science 246 (1990), 1306-1309). This specific endothelial mitogen is upregulated by hypoxia and is able to promote vessel growth when infused into rabbit hindlimbs after femoral artery excision (Takeshita, J. Clin. Invest. 93 (1994), 662-670; Bauters, Am. J. Physiol. 267 (1994), H1263-H1271). These studies however did not distinguish between capillary sprouting, a mechanism called angiogenesis, and true collateral artery growth. Whereas VEGF is only mitogenic for endothelial cells, collateral artery growth requires the proliferation of endothelial and smooth muscle cells and pronounced remodeling processes occur (Schaper, J. Collateral Circulation - Heart, Brain, Kidney, Limbs. Boston, Dordrecht, London: Kluwer Academic Publishers; 1993; Jakeman, J. Clin. Invest. 89 (1992), 244-253; Peters, Proc. Natl. Acad. Sci. USA 90 (1993), 8915-8919; Millauer, Cell 72 (1993), 835-846; Pasyk, Am. J. Physiol. 242 (1982), H1031-H1037). Furthermore mainly capillary sprouting is observed in ischemic territories for example in the pig heart or in rapidly growing tumors (Schaper, J. Collateral Circulation - Heart, Brain, Kidney,

Limbs. Boston, Dordrecht, London: Kluwer Academic Publishers; 1993; Plate, Nature 359 (1992), 845-848; Bates, Curr. Opin. Genet. Dev. 6 (1996), 12-19; Bates, Curr. Opin. Genet. Dev. 6 (1996), 12-19; Görge, Basic Res. Cardiol. 84 (1989), 524-535). True collateral artery growth, however, is temporally and spacially dissociated from ischemia in most models studied (Schaper, J. Collateral Circulation - Heart, Brain, Kidney, Limbs. Boston, Dordrecht, London: Kluwer Academic Publishers; 1993; Paskins-Hurlburt, Circ. Res. 70 (1992), 546-553). Other or additional mechanisms as those described for angiogenesis in ischemic territories are therefore needed to explain collateral artery growth. From previous studies it is known that these collateral arteries grow from preexisting arteriolar connections (Schaper, J. Collateral Circulation - Heart, Brain, Kidney, Limbs. Boston, Dordrecht, London: Kluwer Academic Publishers; 1993).

However, while agents such as VEGF and other growth factors are presently being employed to stimulate the development of angiogenesis after arterial occlusion, such agents are not envisaged as being capable of modulating the growth of preexisting arteriolar connections into true collateral arteries.

Thus, the technical problem of the present invention is to provide pharmaceutical compositions and methods for the modulation of neovascularization and/or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections.

The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the invention relates to a method for enhancing the neovascularization and/or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections comprising contacting an organ, tissue or cells with a colony stimulating factor (CSF) or a nucleic acid molecule encoding said CSF.

The term '"neovascularization" within the meaning of the present invention refers to a review of Sasayama, Circulation Res. 85 (1992), 1197-1204.

For the purpose of the present invention the growth of arteries from preexisting arteriolar connections is also called "arteriogenesis". In particular, "arteriogenesis" is

the in situ growth of arteries by proliferation of endothelial and smooth muscle cells from preexisting arteriolar connections supplying blood to ischemic tissue, tumor or sites of inflammation. These vessels largely grow outside the affected tissue but are much more important for the delivery of nutrients to the ischemic territory, the tumor or the site of inflammation than capillaries sprouting in the diseased tissue by angiogenic processes.

In the context of the present invention the term "colony stimulating factor (CSF)" refers to proteins and peptides which can act on macrophages and which are capable of promoting collateral artery growth by direct activation, proliferation and/or potentiation of the effector functions of resident and newly-recruited macrophages.

Thus, according to the present invention, any CSF or other substances which are functionally equivalent to a CSF, namely which are capable of promoting collateral artery growth can be used for the purpose of the present invention. The action of the CSF employed in the present invention may not be limited to the above-described specificity but they may also act on, for example eosinophils, lymphocyte subpopulations and/or stem cells. Advantageously, the CSF is antiatherogenic.

In accordance with the present invention, it has surprisingly been found that that locally applied Granulocyte-Macrophage-Colony-Stimulating-Factor (GM-CSF) caused a significant increase in collateral artery growth. These results were based on a marked increase of collateral conductance measurements. Peripheral pressures and collateral flows were measured under maximal vasodilation using Statham pressure transducers, fluorescent microspheres and FACS analysis which allowed the calculation of collateral conductances from pressure flow relations. Furthermore, post mortem angiograms revealed a significantly higher number of collateral arteries compared to untreated animals. To the best of the inventors' knowledge, this is the very first report that antiatherogenic and widely clinical established colony stimulating factors are capable of significantly enhancing neovascularization and/or collateral artery growth and/or the growth of other arteries from preexisting arteriolar connections in vivo. Hence, CSFs that can be employed in accordance with the present invention are particularly suited for the treatment of atheriosclerosis.

Experiments performed within the scope of the present invention demonstrate that local infusion of GM-CSF increases both collateral- and peripheral conductance after femoral artery occlusion due to enhanced vessel growth by its proliferative effects on macrophages. Thus, CSFs or nucleic acid molecules encoding CSFs can be used for the activation and proliferation of macrophages which in turn leads to neovascularization and/or the growth of collateral arteries as well as to growth of arteries from preexisting arteriolar connections, which is needed for the cure of several occlusive diseases. Granulocyte colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) belong to a family of glycoprotidic growth factors required for the survival, growth and differentiation of heamatopoietic precursor cells. Therefore this substance has been used clinically to

treat patients with heamatologic and oncologic disorders. The action of these CSF molecules was thought to be restricted to cells of the heamatopoietic origin (Demetri, Semin. Oncol. 19 (1992), 362-385; Lieschke, N. Engl. J. Med. 327(1992), 28-35 /Comments 99-106). Furthermore, several studies have demonstrated that these colony stimulating factors also play a major role in lipid metabolism.

Although recent experiments have shown that GM-CSF is able to directly promote a number of macrophage and granulocyte effector functions including cell survival (Selgas, Kidney International 50 (1996), 2070-2078; Lopez, J. Clin. Invest. 78 (1986), 1220-1228; Eischen, J. Immunol. Meth. 147 (1991), 3408-3412; Vincent, Exp. Hematol. 20 (1992), 17-23; Mangan, J Immunol. 147 (1991), 3408-3412), activation, proliferation (Hoedemakers, Hepatology 13 (1994), 666-674; Matsushime, Japanese Journal of Clinical Hematology 36 (1995), 406-409); differentiation (Munn, Cancer Immunology, Immunotherapy 41 (1995), 46-52), and migration of local tissue macrophages (Bussolini, Nature 337 (1989), 471-473) it was not known that GM-CSF or other colony stimulating factors play a role in the development of collateral arteries and arteriogenesis.

The CSFs to be employed in the methods and uses of the present invention may be obtained from various sources described in the prior art; see, e.g., Gaertner, Bioconjugate Chemistry 3 (1992), 262-268; Dexter, European Journal of Cancer 30A (1994), 15-9; Rohde, Developments in Biological Standardization 83 (1994), 121-127; Lu, Protein Expression & Purification 4 (1993), 465-472; Itoh, Tanpakushitsu Kakusan Koso - Protein, Nucleic Acid, Enzyme 35, 2620-2631. The potential exists, in the use of recombinant DNA technology, for the preparation of various derivatives of colony stimulating factor (CSF) comprising a functional part thereof or proteins which are functionally equivalent to CSFs as described above. In this context, as used throughout this specification "functional equivalent or "functional part" of an CSF means a protein having part or all of the primary structural conformation of a CSF possessing at least the biological property of promoting at least one macrophage or granulocyte effector function mentioned above. The functional part of said protein or the functionally equivalent protein may be a derivative of an CSF by way of amino acid deletion(s), substitution(s), insertion(s), addition(s) and/or

replacement(s) of the amino acid sequence, for example by means of site directed mutagenesis of the underlying DNA. Recombinant DNA technology is well known to those skilled in the art and described, for example, in Sambrook et al. (Molecular cloning; A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY (1989)). Modified CSFs are described, e.g., in Yamasaki, Journal of Biochemistry 115 (1994), 814-819.

CSFs or functional parts thereof or proteins which are functionally equivalent to CSFs, may be produced by known conventional chemical syntheses or recombinant techniques employing the amino acid and DNA sequences described in the prior art; see, e.g., EP-A-0 177 568; Han, Source Gene 175 (1996), 101-104; Kothari, Blood Cells, Molecules & Diseases 21 (1995), 192-200; Holloway, European Journal of Cancer 30A (1994), 2-6. For example, CSFs may be produced by culturing a suitable cell or cell line which has been transformed with a DNA sequence encoding upon expression under the control of regulatory sequences a CSF or a functional part thereof or a protein which is functionally equivalent to CSF. Suitable techniques for the production of recombinant proteins are described in, e.g., Sambrook, supra. Methods for constructing CSFs and proteins as described above useful in the methods and uses of the present invention by chemical synthetic means are also known to those of skill in the art.

In another embodiment, the invention relates to the use of a colony stimulating factor (CSF) or a nucleic acid molecule encoding said CSF for the preparation of a pharmaceutical composition for enhancing neovascularization and/or collateral growth of collateral arteries and/or other arteries from preexisting arteriolar connections.

The pharmaceutical composition comprises at least one CSF as defined above, and optionally a pharmaceutically acceptable carrier or exipient. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by conventional methods. The pharmaceutical compositions can be administered to the subject at a suitable dose. The dosage regimen may be

determined by the attending physician considering the condition of the patient, the severity of the disease and other clinical factors. Administration of the suitable compositions may be effected by different ways, e.g. by intravenous, intraperetoneal, subcutaneous, intramuscular, topical or intradermal administration. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 µg to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1 µg to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately $10^6$ to $10^{12}$ copies of the DNA molecule. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery.

In a preferred embodiment, said CSF used in the methods and uses of the invention is selected from the group consisting of Granulocyte-Macrophage-Colony-Stimulating Factor (GM-CSF), Granulocyte-Colony-Stimulating Factor (G-CSF), Macrophage-Colony-Stimulating Factor (M-CSF), Colony-Stimulating Factor (CSF-I), functionally equivalent substances or functional derivatives thereof.

In a preferred embodiment, the methods and uses of the invention may be employed for diseases caused by a vascular disease or a cardiac infarct or a stroke or for any disease where an increase of blood supply via collaterals, arteries etc. is needed.

In a particularly preferred embodiment, the methods and uses of the invention are designed to be applied to a subject suffering from arteriosclerosis, a coronary artery

disease, a cerebral occlusive disease, a peripheral occlusive disease, a visceral occlusive disease, renal occlusive disease, a mesenterial arterial insufficiency or an ophthamic or retenal occlusion or for any disease where atherosclerotic plaques in the vascular wall lead to an obstruction of the vessel diameter.

In a further preferred embodiment, the methods and uses of the invention are designed to be applied to a subject during or after exposure to an agent or radiation or surgical treatment which damage or destroy arteries.

In a preferred embodiment, the CSF used in the methods and uses of the invention is a recombinant CSF. DNA sequences encoding CSFs which can be used in the methods and uses of the invention are described in the prior art; see, e.g., Holloway, European Journal of Cancer 30A (1994), 2-6 or references cited above. Moreover, DNA and amino acid sequences of CSFs are available in the Gene Bank database. As described above, methods for the production of recombinant proteins are well-known to the person skilled in the art; see, e.g., Sambrook, supra.

In a further preferred embodiment, the method and the use of the present invention is designed to be applied in conjugation with a growth factor, preferably fibroblast growth factor or vascular endothelial growth factor (VEGF). This embodiment is particularly suited for enhancing of both sprouting of capillaries (angiogenesis) and in situ enlargement of preexisting arteriolar connections into true collateral arteries. Pharmaceutical compositions comprising, for example, CSF such as GM-CSF, and a growth factor such as VEGF may be used for the treatment of peripheral vascular diseases or coronary artery disease.

In another preferred embodiment, the method of the invention comprises

(a) obtaining cells, tissue or an organ from a subject;

(b) introducing into said cells, tissue or organ a nucleic acid molecule encoding and capable of expressing the CSF in vivo; and

(c) reintroducing the cells, tissue or organ obtained in step (b) into the same subject or a different subject.

It is envisaged by the present invention that the CSFs and the nucleic acid molecules encoding the CSFs are administered either alone or in combination, and optionally together with a pharmaceutically acceptable carrier or exipient. Said nucleic acid molecules may be stably integrated into the genome of the cell or may be maintained in a form extrachromosomally, see, e.g., Calos, Trends Genet. 12 (1996), 463-466. On the other hand, viral vectors described in the prior art may be used for transfecting certain cells, tissues or organs.

Furthermore, it is possible to use a pharmaceutical composition of the invention which comprises a nucleic acid molecule encoding a CSF in gene therapy. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, adenoviruses, and adeno-associated viruses, among others. Delivery of nucleic acid molecules to a specific site in the body for gene therapy may also be accomplished using a biolistic delivery system, such as that described by Williams (Proc. Natl. Acad. Sci. USA 88 (1991), 2726-2729).

Standard methods for transfecting cells with nucleic acid molecules are well known to those skilled in the art of molecular biology, see, e.g., WO 94/29469. Gene therapy to prevent or decrease the development of diseases described herein may be carried out by directly administering the nucleic acid molecule encoding a CSF to a patient or by transfecting cells with said nucleic acid molecule *ex vivo* and infusing the transfected cells into the patient. Furthermore, research pertaining to gene transfer into cells of the germ line is one of the fastest growing fields in reproductive biology. Gene therapy, which is based on introducing therapeutic genes into cells by ex-vivo or in-vivo techniques is one of the most important applications of gene transfer. Suitable vectors and methods for in-vitro or in-vivo gene therapy are described in the literature and are known to the person skilled in the art; see, e.g., Giordano, Nature Medicine 2 (1996), 534-539; Schaper, Circ. Res. 79 (1996), 911-919; Anderson, Science 256 (1992), 808-813; Isner, Lancet 348 (1996), 370-374; Muhlhauser, Circ. Res. 77 (1995), 1077-1086; Wang, Nature Medicine 2 (1996), 714-716; WO94/29469; WO 97/00957 or Schaper, Current Opinion in Biotechnology 7 (1996), 635-640, and references cited therein. The nucleic acid molecules comprised

in the pharmaceutical composition of the invention may be designed for direct introduction or for introduction via liposomes, or viral vectors (e.g. adenoviral, retroviral) containing said nucleic acid molecule into the cell. Preferably, said cell is a germ line cell, embryonic cell, or egg cell or derived therefrom.

It is to be understood that the introduced nucleic acid molecules encoding the CSF express said CSF after introduction into said cell and preferably remain in this status during the lifetime of said cell. For example, cell lines which stably express said CSF may be engineered according to methods well known to those skilled in the art. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the recombinant DNA molecule or vector of the invention and a selectable marker, either on the same or separate vectors. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows for the selection of cells having stably integrated the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express a CSF. Such cells may be also be administered in accordance with the pharmaceutical compositions, methods and uses of the invention.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, Cell 11(1977), 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska, Proc. Natl. Acad. Sci. USA 48 (1962), 2026), and adenine phosphoribosyltransferase (Lowy, Cell 22 (1980), 817) in $tk^-$, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, Proc. Natl. Acad. Sci. USA 77 (1980), 3567; O'Hare, Proc. Natl. Acad. Sci. USA 78 (1981), 1527), gpt, which confers resistance to mycophenolic acid (Mulligan, Proc. Natl. Acad. Sci. USA 78 (1981), 2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, J. Mol. Biol. 150 (1981), 1); hygro, which confers resistance to hygromycin (Santerre, Gene 30 (1984), 147); or puromycin (pat, puromycin N-acetyl transferase). Additional selectable genes have been described, for example, trpB,

which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

Thus, in a preferred embodiment, the nucleic acid molecule comprised in the pharmaceutical composition for the use of the invention is designed for the expression of the CSF by cells in vivo by, for example, direct introduction of said nucleic acid molecule or introduction of a plasmid, a plasmid in liposomes, or a viral vector (e.g. adenoviral, retroviral) containing said nucleic acid molecule.

In a preferred embodiment of the method and uses of the present invention, the CSF derivative or functional equivalent substance is an antibody, (poly)peptide, nucleic acid, small organic compound, ligand, hormone, PNA or peptidomimetic.

In this context, it is understood that the CSFs to be employed according to the present invention may be, e.g., modified by conventional methods known in the art. For example, it is possible to use fragments which retain the biological activity of CSFs as described above, namely the capability of promoting collateral artery growth. This further allows the construction of chimeric proteins and peptides wherein other functional amino acid sequences may be either physically linked by, e.g., chemical means to the CSF or may be fused by recombinant DNA techniques well known in the art. Furthermore, folding simulations and computer redesign of structural motifs of the CSFs or their receptors can be performed using appropriate computer programs (Olszewski, Proteins 25 (1996), 286-299; Hoffman, Comput. Appl. Biosci. 11 (1995), 675-679). Computer modeling of protein folding can be used for the conformational and energetic analysis of detailed receptor and protein models (Monge, J. Mol. Biol. 247 (1995), 995-1012; Renouf, Adv. Exp. Med. Biol. 376 (1995), 37-45). In particular, the appropriate programs can be used for the identification of interactive sites of the CSF and its receptor by computer assistant searches for complementary peptide

sequences (Fassina, Immunomethods 5 (1994), 114-120). Further appropriate computer systems for the design of protein and peptides are described in the prior art, for example in Berry, Biochem. Soc. Trans. 22 (1994), 1033-1036; Wodak, Ann. N. Y. Acad. Sci. 501 (1987), 1-13; Pabo, Biochemistry 25 (1986), 5987-5991. The results obtained from the above-described computer analysis can be used for, e.g., the preparation of peptidomimetics of the CSFs or fragments thereof. Such pseudopeptide analogues of the natural amino acid sequence of the protein may very efficiently mimic the parent protein or peptide (Benkirane, J. Biol. Chem. 271 (1996), 33218-33224). For example, incorporation of easily available achiral Ω-amino acid residues into a CSF protein or a fragment thereof results in the substitution of amide bonds by polymethylene units of an aliphatic chain, thereby providing a convenient strategy for constructing a peptidomimetic (Banerjee, Biopolymers 39 (1996), 769-777). Superactive peptidomimetic analogues of small peptide hormones in other systems are described in the prior art (Zhang, Biochem. Biophys. Res. Commun. 224 (1996), 327-331). Appropriate peptidomimetics of CSF may also be identified by the synthesis of peptidomimetic combinatorial libraries through successive amide alkylation and testing the resulting compounds, e.g., according to the methods described in the prior art. Methods for the generation and use of peptidomimetic combinatorial libraries are described in the prior art, for example in Ostresh, Methods in Enzymology 267 (1996), 220-234 and Dorner, Bioorg. Med. Chem. 4 (1996), 709-715. Furthermore, antibodies or fragments thereof may be employed which, e.g., upon binding to a CSF-receptor mimic the biological activity of a CSF.

Furthermore, a three-dimensional and/or crystallographic structure of the CSF or of its receptor can be used for the design of peptidomimetic inhibitors of the biological activity of a CSF (Rose, Biochemistry 35 (1996), 12933-12944; Rutenber, Bioorg. Med. Chem. 4 (1996), 1545-1558).

As discussed above, neovascularization and the growth of arteries from preexisting arteriolar connections is essential for the delivery of nutrition to tumors. Thus, if the growth of said vessels to the tumor would be suppressed suppression and/or inhibition of tumor growth is to be expected.

Accordingly, the present invention also relates to a method for the treatment of tumors comprising contacting an organ, tissue or cells with an agent which suppresses neovascularization and/or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections through the inhibition of the biological activity of a CSF.

Tumor Macrophages require specific growth factors, e.g., M-CSF/CSF-1, for their proliferation throughout the G1 phase of the cell cycle. Once cells enter S phase, macrophages complete mitosis in the absence of M-CSF/CSF-1. During the G1 phase, cyclin D (a cell cyclus regulator, that together with cyclin dependent kinase (cdk 4) promotes entry of the cell into M-phase (Alberts, Biology of the Cell (1989), Second Edition) is induced by M-CSF/CSF-1 stimulation. The enzymatic activity of cyclin D could be negatively regulated by recently reported inhibitory proteins to determine the timing for entry into S phase in macrophages (Matsushime, Japanese Journal of Clinical Hematology 36 (1995), 406-409).

It could be shown that among CSF-dependent macrophages especially monocytes as well as tissue specific macrophages (in the female reproductive tract) seem to be dependent on CSF-1 for their further differentiation (Maito, Mol. Reprod. Dev. 46 (1997), 85-91). Beyond this GM-CSF/M-CSF are essential for the macrophage survival. Thus, as it could be demonstrated in accordance with the present invention that CSFs promote neovascularization and collateral artery growth withdrawal of these factors should result in inhibition or decrease of neovascularization and/or collateral artery growth and, thus, in the suppression of tumor growth. Agents which suppress neovascularization and/or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections may be peptides, proteins, nucleic acids, antibodies, small organic compounds, hormones, neural transmitters, peptidomimics, or PNAs (Milner, Nature Medicine 1 (1995), 879-880; Hupp, Cell 83 (1995), 237-245; Gibbs, Cell 79 (1994), 193-198). For the preparation and application of such compounds, the person skilled in the art can use the methods known in the art, for example those referred to above.

The present invention further relates to the use of an agent which suppresses neovascularization and/or the growth of collateral arteries and/or other arteries from

preexisting arteriolar connections through the inhibition of the biological activity of a CSF for the preparation of a pharmaceutical composition for the treatment of tumors.

In a preferred embodiment, the agent used in the methods and uses of the invention as described above inhibits the biological activity of a CSF and/or inhibits an intracellular signal or signal cascade comprising MAPK and/or JNK/SAPK triggered in macrophages through the receptor for the CSF. Various receptors of CSFs are described in the prior art, for example in Chemokine Receptors. Immunology Today (1996), Suppl S: 26-27; Bendel, Leukemia & Lymphoma 25 (1997), 257-270; Perentesis, Leukemia & Lymphoma 25 (1997), 247-256; Bishay, Scandinavian Journal of Immunology 43 (1996), 531-536; Kluck, Annals of Hematology 66 (1993), 15-20; Raivich, Journal of Neuroscience Research 30 (1991), 682-686 or in Wong, Cellular Immunology 123 (1989), 445-455.

In another preferred embodiment, said receptor is a CSF receptor. Said receptor or specific domains thereof which a responsible for triggering a signal leading to collateral artery growth may be blocked or modulated by methods described herein.

In a preferred embodiment, the agent used in the methods and uses of the invention is a(n) antibody, (poly)peptide, nucleic acid, small organic compound, ligand, hormone, PNA or peptidomimetic.

Nucleic acid molecules specifically hybridizing to CSF encoding genes and/or their regulatory sequences may be used for repression of expression of said gene, for example due to an antisense or triple helix effect or they may be used for the construction of appropriate ribozymes (see, e.g., EP-B1 0 291 533, EP-A1 0 321 201, EP-A2 0 360 257) which specifically cleave the (pre)-mRNA of a gene encoding a CSF. The nucleic and amino acid sequences encoding CSFs are known in the art and described, for example, in Han, Source Gene 175 (1996), 101-104; Kothari, Blood Cells, Molecules & Diseases 21 (1995), 192-200 or in Holloway, European Journal of Cancer 30A (1994), 2-6. Selection of appropriate target sites and corresponding ribozymes can be done as described for example in Steinecke,

Ribozymes, Methods in Cell Biology 50, Galbraith et al. eds Academic Press, Inc. (1995), 449-460.

Nucleic acids comprise DNA or RNA or hybrids thereof. Furthermore, said nucleic acid may contain, for example, thioester bonds and/or nucleotide analogues, commonly used in oligonucleotide anti-sense approaches. Said modifications may be useful for the stabilization of the nucleic acid molecule against endo- and/or exonucleases in the cell. Furthermore, the so-called "peptide nucleic acid" (PNA) technique can be used for the inhibition of the expression of a gene encoding a CSF. For example, the binding of PNAs to complementary as well as various single stranded RNA and DNA nucleic acid molecules can be systematically investigated using, e.g., thermal denaturation and BIAcore surface-interaction techniques (Jensen, Biochemistry 36 (1997), 5072-5077). The synthesis of PNAs can be performed according to methods known in the art, for example, as described in Koch, J. Pept. Res. 49 (1997), 80-88; Finn, Nucleic Acids Research 24 (1996), 3357-3363. Furthermore, folding simulations and computer redesign of structural motifs of the CSFs and their receptors can be performed as described above to design drugs capable of inhibiting the biological activity of CSFs.

Furthermore, antibodies may be employed specifically recognizing CSF or their receptors or parts, i.e. specific fragments or epitopes, of such CSFs and receptors thereby inactivating the CSF or the CSF receptor. These antibodies can be monoclonal antibodies, polyclonal antibodies or synthetic antibodies as well as fragments of antibodies, such as Fab, Fv or scFv fragments etc. Antibodies or fragments thereof can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988 or EP-B1 0 451 216 and references cited therein. For example, surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage antibodies which bind to an epitope of the CSF or its receptor (Schier, Human Antibodies Hybridomas 7 (1996), 97-105; Malmborg, J. Immunol. Methods 183 (1995), 7-13).

Putative inhibitors which can be used in accordance with the present invention including peptides, proteins, nucleic acids, antibodies, small organic compounds, ligands, hormones, peptidomimetics, PNAs and the like capable of inhibiting the

biological activity of a CSF or its receptor may be identified according to the methods known in the art, for example as described in EP-A-0 403 506 or in the appended examples.

In a preferred embodiment, the agent which blocks the interaction of the CSF and its receptor is selected from the group consisting of

(i) an anti-CSF antibody and an anti-CSF-receptor antibody; and/or

(ii) a non-stimulatory form of a CSF protein and a soluble form of a CSF-receptor.

Such antibodies as well as inactive and soluble forms of CSFs and their receptors, respectively, are described in, e.g., Kogut, Inflammation 21 (1997) or in Shimamura, Journal of Histochemistry & Cytochemistry 38 (1990), 283-286 and can be obtained according to methods known in the art; see, e.g., supra.

In a preferred embodiment of the present invention, the agent is designed to be expressed in vascular cells or cells surrounding preexisting arteriolar connections to a tumor.

In a preferred embodiment, methods and uses of the invention are employed for the treatment of a tumor which is a vascular tumor, preferably selected from the group consisting of Colon Carcinoma, Sarcoma, Carcinoma in the breast, Carcinoma in the head/neck, Mesothelioma, Glioblastoma, Lymphoma and Meningeoma.

In a preferred embodiment, the pharmaceutical composition in the use of the invention is designed for administration by catheter intraarterial, intravenous, intraperitoneal or subcutenous routes. In the examples of the present invention the CSF protein was administered locally via osmotic minipump.

These and other embodiments are disclosed or are obvious from and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on Internet, e.g. under

http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The use and methods of the invention can be used for the treatment of all kinds of diseases hitherto unknown as being related to or dependent on the modulation of neovascularization and/or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections. The methods and uses of the present invention may be desirably employed in humans, although animal treatment is also encompassed by the methods and uses described herein.

The figures show

Figure 1: Angiography of the whole right leg of an animal treated with GM-CSF.

Figure 2: Angiography of the whole right leg (A) and of the collateral circulation (B) (without Os femoris) of an animal treated with GM-CSF.

Figure 3: Angiography of the collateral circulation (without Os femoris) of an animal treated with GM-CSF.

Figure 4: Angiography of the whole right leg of an animal treated with PBS.

Figure 5: Angiography of the collateral circulation (without Os femoris) of an animal treated with PBS.

The examples illustrate the invention.

**Example 1: Femoral artery occlusion of animals and local delivery of agents**

The present study was performed with the permission of the State of Hessen, Regierungspräsidium Darmstadt, according to section 8 of the *German Law for the Protection of Animals*. It confirms with the *Guide for the Care und Use of Laboratoy Animals published by the US National Institut of Health* (NIH Publication No. 85-23, revised 1985).

6 rabbits were subjected to 7 days of right femoral artery occlusion. They were randomly assigned to either receive GM-CSF (Novartis, Nuernberg, Germany) (2ML-2, Alza Corp; 3 µg in 2 mL PBS at a rate of 10 µL/h) or PBS locally via osmotic minipump. For the initial implantation of the osmotic minipumps, the animals were anesthetized with an intramuscular injection of ketamine hydrochloride (40 to 80 mg/kg body weight) and xylazine (8 to 9 mg/kg body weight). Supplementary doses of anesthetic (10% to 20% of the initial dose) were given intravenously as needed. The surgical procedure was performed under sterile conditions. Femoral arteries were exposed and cannulated with a sterile polyethylene catheter (inner diameter: 1mm; outer diameter: 1,5mm) pointing upstream, with the tip of the catheter positioned distal to the branching of the arteria circemflexa femoris. The catheter itself was connected to the osmotic minipump (2ML-2, Alza Corp), which was implanted under the skin of the lower right abdomen. After that the animals were outfitted with a specially designed body suit that allowed them to move freely but prevented self-mutilation. The rabbits were housed individually with free access to water and chow to secure mobility. The body weights and body temperature in rabbits treated with GM-CSF did not significantly differ from those of control rabbits. Serum values of total protein, albumin, glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase were not significantly changed by the GM-CSF treatment.

Seven days after implantation the animals were again anasthetized with an intramuscular injection of ketamine hydrochloride and xylazine for tracheostomy and artificial ventilation. Anesthesia was deepened with pentobarbital (12 mg/kg body

weight per hour). The carotid artery was cannulated for continuous pressure monitoring. The arteria saphena magna (anterior tibial artery in humans and main arterial supply to the lower limb and foot in the rabbit) was exposed just above the ankle and cannulated with sterile polyethylene heparinized tubing (inner diameter 0,58 mm; outer diameter 0,9 6mm). They were connected to a Statham P23DC pressure transducer (Statham, Spectramed) for measurement of peripheral pressures (PP). After heparinization with 5000 Units heparin, the left femoral artery was exposed and cannulated with sterile polyethylene catheter (inner diameter: 1 mm; outer diameter: 1,5 mm) for the microsphere reference sample. After cannulation of the abdominal aorta a shunt was installed to ensure oxygenated blood flow from the carotid artery via the canula in the abdominal aorta into the right and left legs. A flow probe was installed to measure total flow to both hindlimbs.

**Example 2: Ex vivo pressure-flow relations**

Maximum vasodilation was achieved by injecting 20 mg papaverine (Sigma) to the shunt at a flow rate of 20 ml/min. After stabilization of peripheral and central pressures both legs were perfused via four different pressure. Each pressure gradient was combined with a bolus of microspheres.

Five different perfusion pressures (30,40,50,60,80 mmHg) were generated in vivo with a roller pump installed in the above mentioned shunt between carotid artery and abdominal aorta. Peripheral pressures and collateral flows were measured under maximal vasodilation (papaverine) using Statham pressure transducers.

For each pressure level microspheres with a different fluorescent color (either crimson, scarlett, blue-green, red or blue) were injected into the mixing chamber, which was installed in the carotid-abdominal aortic shunt.

The following muscles were dissected from the leg: Quadriceps, adductor longus, adductor magnus, gastrocnemius, soleus, and peroneal muscles. Each muscle was divided into 3 three consecutive samples from the proximal to the distal end. The whole muscle and afterwards each sample were weighed and cut to small pieces. The muscle sample were then placed loosely into 12 mm x 75 mm polystyrene tubes

(Becton Dickinson & Co, Lincoln Park, NJ) and 3 ml of SDS solution [SDS solution (Boehringer Mannheim Corp.): 1% SDS (Boehringer Mannheim Corp.), 0,5% sodium azide (Sigma Chemical Company, St. Louis, MO), and 0,8% Tween-80 (Fisher Scientific, Fairlawn, NJ) in 50 millimolar pH 8 tris buffer (Sigma Chemical Company, St. Louis, MO)], 30 µl proteinase K solution (Boehringer Mannheim Corp.) and 1 ml of microspheres as internal standard was added (13,7 µm, Fluorescein Kit, Flow Cytometry Standards, Corp. San Juan, P.R.). Each tube was capped and secured in a shaking water bath for 24-48 hours. The samples were then subsequently spinned at 1000g for 45 minutes, the supernatant was pipetted off and the pellet was resuspended in 1 ml PBS (pH 7.4). Before FACS analysis the probes were rigorously shaken. The microspheres were counted using a flow cytometer (FACS-Calibur) equipped with a second laser and a detector for a fourth fluorescence. Flows for each sample were calculated from the number of microspheres in the sample ($m^S$), the respective microspheres count in the reference sample ($m^{rs}$), the internal standard in the sample (ISs), internal standard in the reference sample (IS rs), the weight of the reference sample (W) and the time during which the reference sample was withdrawn using following equation.

$$\text{flow [mg/ml]} = \frac{m^s \cdot IS^{rs}}{IS^s \cdot m^{rs}} \cdot \frac{w}{t}$$

$m^s$ = sample microsphere
$IS^{rs}$ = internal standard reference sample
$IS^s$ = internal standard sample
$m^{rs}$ = microsphere reference sample
w = weight
t = time

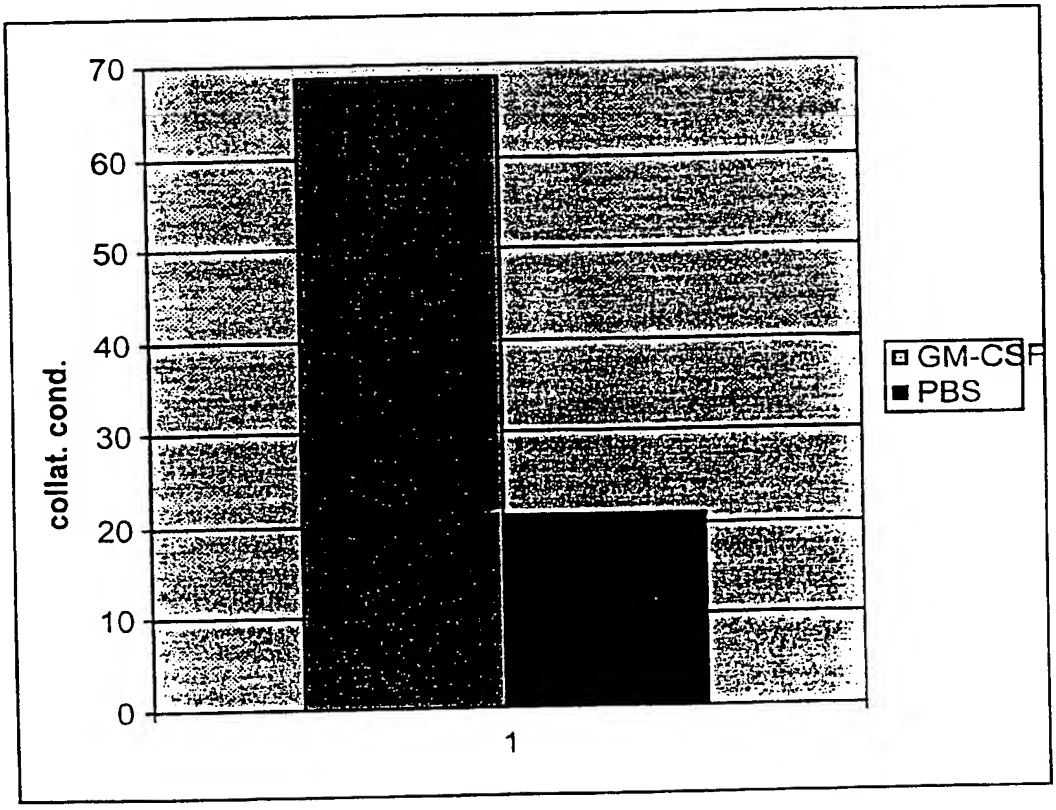
In the present model, collateral arteries developing after femoral artery occlusion in typical corkscrew formation supply blood to the distal adductor region and the lower leg. The systemic pressure [SP] and peripheral pressure [PP] was measured.

Venous pressure was equal to atmospheric pressure [AP] (zero in the present case). Since arterial resistances are much lower than collateral and peripheral resistances, they can be neglected. SP represent the pressure at the stem region of the collateral arteries. PP is the pressure at the reentry region and is identical to the pressure head of the circulation in lower leg; AP, the pressure at the venous end of the peripheral circulation. Collateral flow is equal to the sum of flow to the tissue of the distal adductor plus the flow to the tissue of the lower leg. Collateral resistance was defined as pressure difference between SP and PP divided by the flow going to the distal adductor an the lower leg. Peripheral resistance was defined as PP divided by flow to the lower leg, and bulk conductance was defined as SP divided by bulk flow recorded with the ultrasonic flow probe. The reciprocal values of these resistances represent collateral, peripheral, and bulk conductance. Because a positive pressure intercept is observed event at maximal vasodilation, all conductances were calculated from the slope of pressure-flow relations. Data are described as mean $\pm$ SD. Differences among data were assessed using unpaired Student's t-test for intergroup comparisons and Mann-Whithney rank-sum test for unequal variances. Values of $p \leq .05$ were required for assumption of statistical significance. Collateral conductance was significantly higher after 1 week of occlusion in animals treated with GM-CSF compared with animals without this treatment.

Table 1

## collateral conductance [ml/min/100mmHg]

| | GM-CSF | PBS | p |
|---|---|---|---|
| mean | 68,685 | 21,101 | 0.001 |



**Example 3: Post mortem angiography**

Legs were perfused with Krebs-Henseleit buffered saline in a warmed waterbath of 37°C for 1 minute at a pressure of 80 mmHg, followed by perfusion with contrast medium (8 to 10 minutes at 80 mmHg) based on bismuth and gelatin according to a formula developed by Fulton (Fulton: The Coronary Arteries, Thomas Books, 1965). Subsequently, the contrast medium was allowed to gel by placing the limbs on crushed ice for 45 minutes. Angiograms were taken at two different angles in a Balteau radiography apparatus (Machlett Laboratories) using a single-enveloped Structurix D7DW film (AGFA). The resulting stereoscopic pictures allowed analysis of collateral growth in three dimensions.

To differentiate between collateral vessels and muscle vessels for further quantification, Longland's definition of collateral arteries was used (Longland et.al. 1954 "Description of collateral arteries" Verlag: Thomas). Stem, midzone and re-entry were identified under stereoscopic viewing using a 3-fold magnification of our angiograms. Collateral arteries then were divided in two groups: group one consisted of vessels whose stem branched from the Arteria circumflexa femoris lateralis. Group two of the arteries originated from the Arteria profunda femoris. The length of the midzone in each group was almost the same, so their measurement did not give any further information. Re-entry of the collaterals from the first group usually descended into the Arteria genus descendens, the second group into the Arteria caudalis femoris. Only about 10 % of the collateral arteries originate from other vessels, e.g from the A. iliaca externa or from the A. iliaca interna.

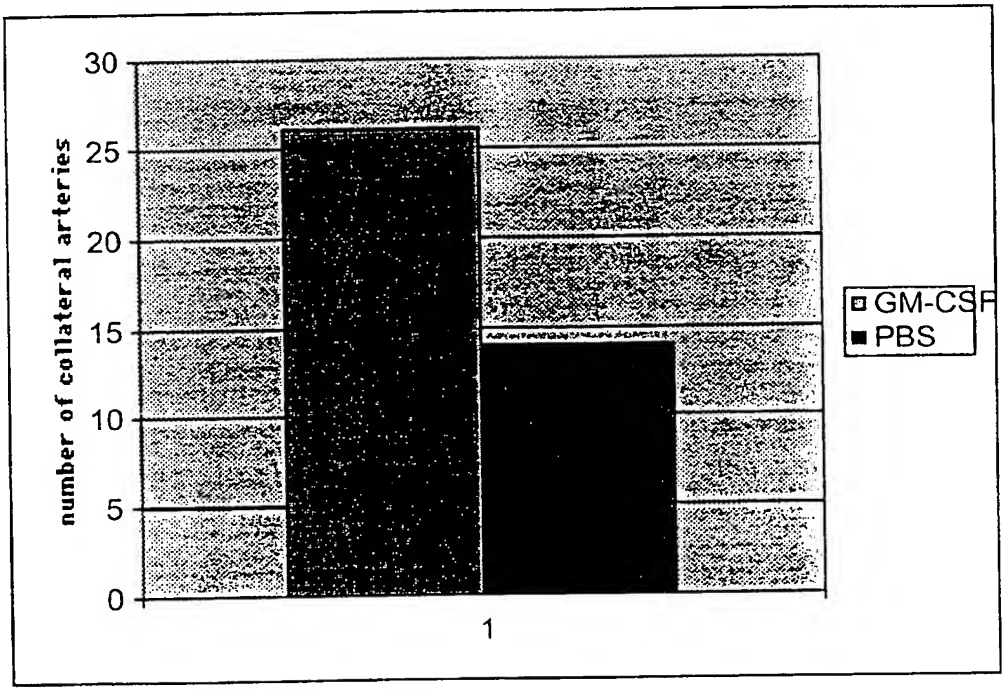
Collateral vessels were marked after counting to make sure, that no vessel was counted twice. A further 3-fold magnification was used to measure the diameter of the vessels with an accuracy of 0.1 mm. Postmortem angiograms exhibited corkscrew collaterals mainly in the adductor longus, adducotr magnus, and vasuts intermedius connecting the perusion bed of the arteria femoralis profunda to that of the arteria saphene parva int the adductor muscles and the perfusion bed of the arteria circumflexa femoris lateralis to that of the arteriae genuales in the quadriceps muscle. Angiograms taken from hindlimsb of animals treated with GM-CSF show a

remarkable increase the diameter and density of these collateral vessels. (Table 2, Figures 1 to 5)

**Table 2**

**collateral arteries**

| | GM-CSF | PBS | p |
|---|---|---|---|
| mean | 26 | 14 | 0,02 |



The results of the experiments performed in accordance with the present invention indicate that CSFs are capable of mediating neovascularization and/or collateral artery growth and/or growth of arteries from preexisting arteriolar connections due to macrophage recruitment that might be mediated by a direct effect of CSFs on macrophage activation, proliferation, motility, and survival and, secondarily, by chemoattractant molecules released in response to locally administered CSFs. Thus, the present invention provides for novel means and methods for the treatment of diseases which depend on neovascularization and/or collateral artery growth.

The present invention is not to be limited in scope by its specific embodiments described which are intended as single illustrations of individual aspects of the invention and any proteins, nucleic acid molecules, or compounds which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those shown and described therein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Said modifications intended to fall within the scope of the appended claims. Accordingly, having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited to particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope of the present invention.

# Claims

1. A method for enhancing neovascularization and/or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections comprising contacting organs, tissue or cells with a colony stimulating factor (CSF) and/or a nucleic acid molecule encoding said CSF.

2. Use of a colony stimulating factor (CSF) and/or a nucleic acid molecule encoding said CSF for the preparation of a pharmaceutical composition for enhancing neovascularization and/or collateral growth of collateral arteries and/or other arteries from preexisting arteriolar connections.

3. The method of claim 1 or the use of claim 2, wherein said CSF is selected from the group consisting of Granulocyte-Macrophage-Colony-Stimulating Factor (GM-CSF), Granulocyte-Colony-Stimulating Factor (G-CSF), Macrophage-Colony-Stimulating Factor (M-CSF), Colony-Stimulating Factor (CSF-I), functionally equivalent substances or functional derivatives thereof.

4. The method of claim 1 or 3 or the use of claim 2 or 3, wherein said method or said pharmaceutical composition is designed to be applied to a subject suffering from a vascular disease or a cardiac infarct or a stroke.

5. The method or the use of claim 4, wherein said vascular disease is arteriosclerosis and/or a hyperlipidemic condition, a coronary artery disease, cerebral occlusive disease, peripheral occlusive disease, visceral occlusive disease, renal artery disease, mesenterial arterial insufficiency or an ophtamic or retenal occlusion.

6. The method of claim 1 or 3 or the use of claim 2 or 3, wherein said method or said pharmaceutical composition is designed to be applied to a subject during or after exposure to an agent or radiation or surgical treatment which damage or destroy arteries.

7. The method of any one of claims 1 or 3 to 6 or the use of any one of claims 2 to 6, wherein the CSF is a recombinant CSF.

8. The method of any one of claims 1 or 3 to 7, further comprising contacting the organ, tissue or cell with a growth factor.

9. The use of any one of claims 2 to 7, wherein the pharmaceutical composition is designed for administration in conjugation with a growth factor.

10. The method of any one of claims 1 or 3 to 8, comprising
(a) obtaining cells, tissue or an organ from a subject;
(b) introducing into said cells, tissue or organ a nucleic acid molecule encoding and capable of expressing the CSF in vivo; and
(c) reintroducing the cells, tissue or organ obtained in step (b) into the same subject or a different subject.

11. The method of any one of claims 1, 2 to 8 or 10 or the use of any one of claims 2 to 7 or 9, wherein the CSF derivative or functional equivalent substance is an antibody, (poly)peptide, nucleic acid, small organic compound, ligand, hormone, PNA or peptidomimetic.

12. A method for the treatment of tumors comprising contacting organs, tissue or cells with an agent which suppresses neovascularization and/or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections through inhibition of the biological activity of a CSF as defined in any one of claims 1 to 11.

13. Use of an agent which suppresses the growth of collateral arteries and/or other arteries from preexisting arteriolar connections through the inhibition of the biological activity of a CSF as defined in any one of claims 1 to 11 for the preparation of a pharmaceutical composition for the treatment of tumors.

14. The method of claim 12 or the use of claim 13, wherein the agent inhibits the biological activity of a CSF and/or inhibits an intracellular signal or signal cascade comprising MAPK and/or JNK/SAPK triggered in macrophages through the receptor for the CSF.

15. The method or the use of claim 14, wherein the agent blocks an interaction of the CSF and its receptor.

16. The method or the use of claim 15, wherein the receptor is a CSF-receptor.

17. The method of claim 12, 14 or 15 or the use of any one of claims 13 to 15, wherein the agent is derived from a class of substances as defined in claim 11.

18. The method or the use of claim 17, wherein the agent is designed to be expressed in vascular cells or cells surrounding preexisting arteriolar connections to a tumor.

19. The method or the use of any one of claims 16 to 18, wherein the agent which blocks an interaction of the CSF is
(i) an anti-CSF antibody or an anti-CSF-receptor antibody; and/or
(ii) a non-stimulatory form of a CSF or a soluble form of a CSF-receptor.

20. The method of any one of claims 12 or 14 to 19 or the use of any one of claims 13 to 19, wherein the tumor is a vascular tumor.

21. The method or the use claim 20, wherein the tumor is selected form the group consisting of Colon Carcinoma, Sarcoma, Carcinoma in the breast, Carcinoma in the head/neck, Mesothelioma, Glioblastoma, Lymphoma and Meningeoma.

22. The use of any one of claims 2 to 7, 9 or 13 to 20, wherein the pharmaceutical composition is designed for administration by intracoronary, intramuscular, intraarterial, intravenous, intraperitoneal or subcutenous routes.

Figure 1

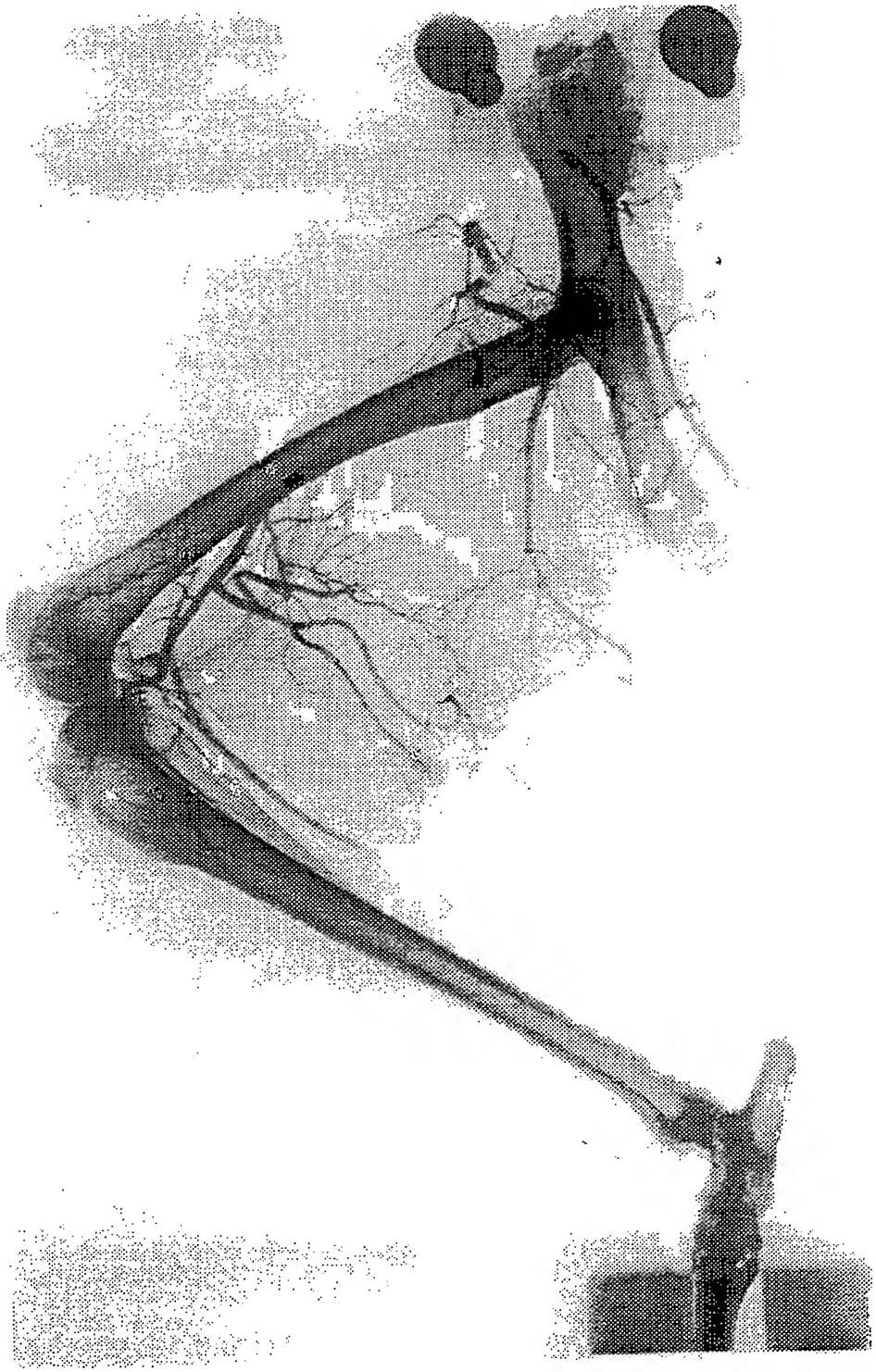


Figure 2A

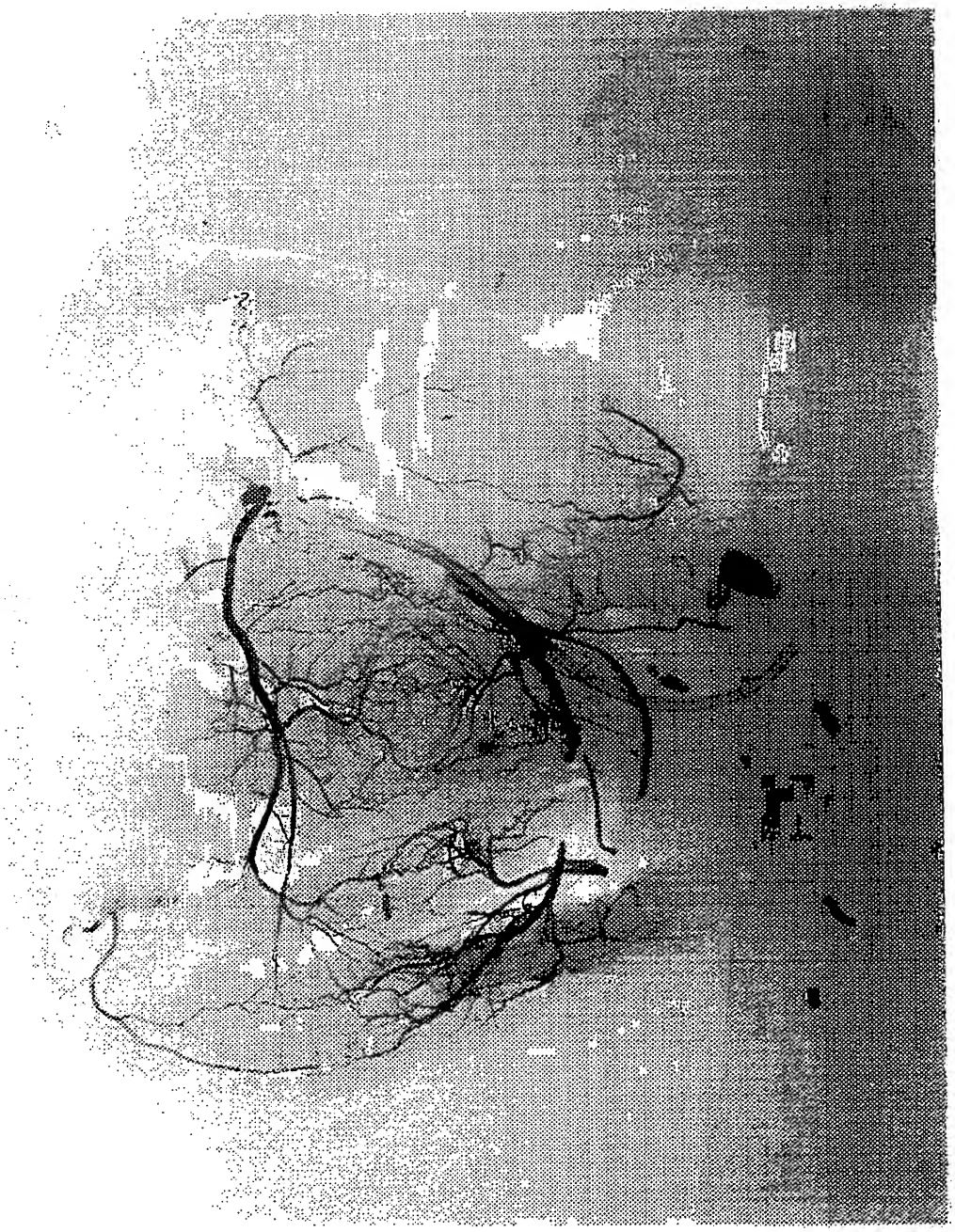


Figure 2B

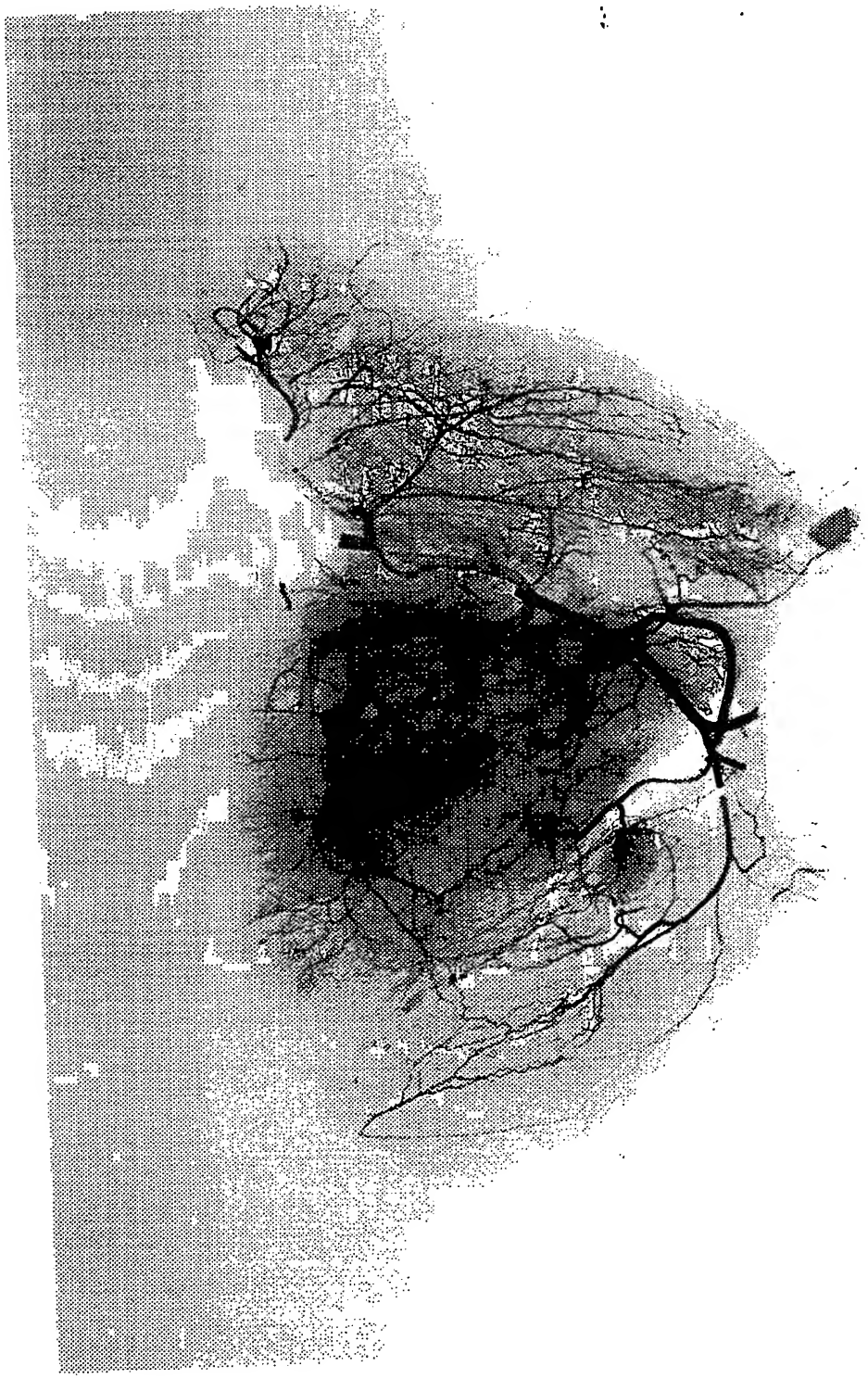


Figure 3

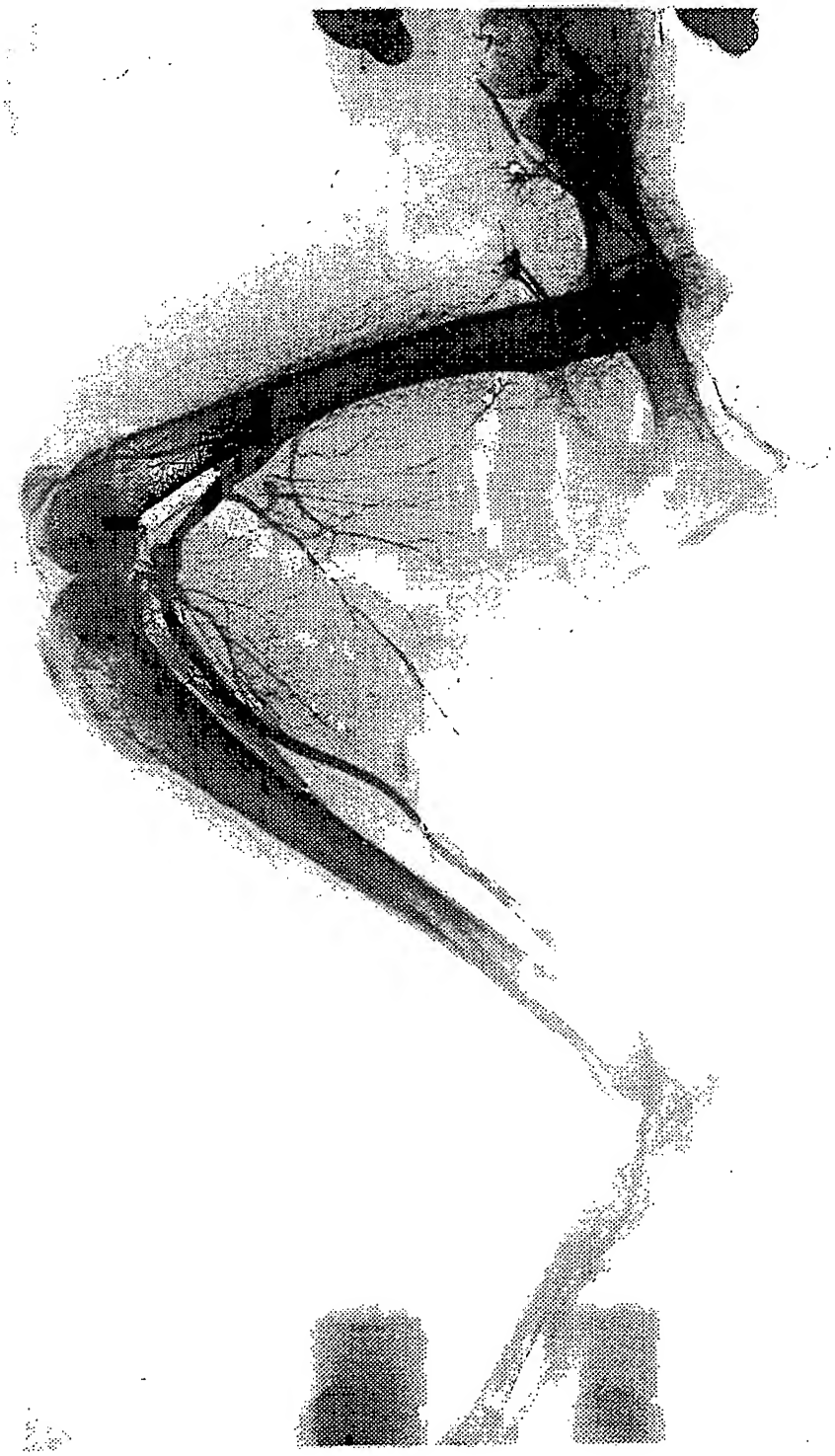


Figure 4

Figure 5

# INTERNATIONAL SEARCH REPORT

nternational Application No
PCT/EP 98/06233

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/19 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 95 21864 A (LUDWIG INST CANCER RES ;NICHOLSON SANDRA ELAINE (AU); LAYTON JUDIT) 17 August 1995 see page 4, line 14 - page 9, line 29 --- | 12-22 |
| A | AHARINEJAD S ET AL: "CSF -1 treatment promotes angiogenesis in the metaphysis of osteopetrotic (toothless, tl) rats." BONE, (1995 MAR) 16 (3) 315-24. JOURNAL CODE: ASR. ISSN: 8756-3282., XP002094806 United States see the whole document --- -/-- | 1-11 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 25 February 1999 | 15/03/1999 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Sitch, W |

1

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/06233

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | ITO W D ET AL: "MONOCYTE CHEMOTACTIC PROTEIN-1 INCREASES COLLATERAL AND PERIPHERAL CONDUCTANCE AFTER FEMORAL ARTERY OCCLUSION" CIRCULATION RESEARCH, vol. 80, no. 6, June 1997, pages 829-837, XP002074060 see page 829 see abstract --- | |
| P,X | BUSCHMANN, IVO ET AL: "GM- CSF promotes collateral artery growth via prolongation of macrophage survival." JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY, (JUNE, 1998) VOL. 30, NO. 6, PP. A126. MEETING INFO.: XVI WORLD CONGRESS OF THE INTERNATIONAL SOCIETY FOR HEART RESEARCH: CARDIOVASCULAR BIOLOGY AND MEDICINE INTO THE 21ST CENTURY ISSN: 0022-2828., XP002094807 see abstract 494 ----- | 1-11 |

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 98/ 06233

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1, 3-8, 11, 12, 14-21 partially and claim 10 completely are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

ternational Application No
PCT/EP 98/06233

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9521864 A | 17-08-1995 | AU 700798 B | 14-01-1999 |
| | | AU 1915395 A | 29-08-1995 |
| | | CA 2182494 A | 17-08-1995 |
| | | EP 0749445 A | 27-12-1996 |
| | | JP 9508799 T | 09-09-1997 |